DRUG ANALYSIS BY MASS SPECTROMETRY

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ABSTRACT

1. The pharmacokinetics of morphine have been measured in four patients with renal failure and three healthy volunteers following intramuscular administration of papaveretum. Morphine blood levels were determined using GCMS with specific ion monitoring. The significantly shorter drug elimination half-life found for the patients suggests that renal failure does not impair the elimination of morphine.

2. A CI GCMS assay with specific ion monitoring has been developed for measuring the antiparkinsonian drug benztropine in post-mortem specimens. The assay is potentially more sensitive than a similar assay using electron impact ionisation.

3. The level of atropine in an aqueous extract of Datura stramonium has been measured by GCMS with selected ion monitoring. The results show that such an extract will contain most of the atropine present in the plant material. The levels are such that several medium-sized glassfuls will contain sufficient atropine to constitute a dangerous drug dose.

4. A Hewlett-Packard 5982A GCMS has been successfully modified to allow it to be used to record in-beam

mass spectra with a commercially available DCI probe. The effectiveness of these modifications is discussed and in-beam mass spectra of some physiologically active compounds are presented.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Drug Analysis By Mass Spectrometry

Mass spectrometry is a powerful instrumental method for chemical analysis. The information contained in a single mass spectrum, recorded using very small amounts of sample, will often allow the unambiguous identification or structural elucidation of an unknown compound. The development of ionisation techniques which remove the requirement that the sample be vaporised before ionisation has made a whole range of compounds of biomedical and biochemical importance amenable to mass spectrometric analysis.

The high sensitivity and specificity of the mass spectrometer are enhanced by using a gas chromatograph as the mass spectrometer inlet, and combined gas chromatography-mass spectrometry (GCMS) is one of the most powerful analytical techniques available today. The combination of the very high sensitivity and specificity of GCMS is unobtainable even with highly sensitive immunological techniques. A number of major developments have occurred in recent years in GCMS methodology. These include the direct coupling of capillary gas chromatographic columns to mass spectrometers, the widespread availability of ion sources for chemical ionisation, and the increasing use of isotopically labelled internal standards in assays using quantitative selected ion monitoring (QSIM). Capillary columns offer two main advantages over packed columns. An assay sensitivity is greatly increased by using

a capillary column, and separations of components in a complex mixture that were previously unobtainable with packed columns can often be performed almost routinely. Chemical ionisation (CI), although it has some disadvantages, is well suited for many analyses. The intense peaks usually seen in the molecular ion region of a CI spectrum will often allow rapid identification of an unknown compound. The ions producing these peaks can also be monitored in a CI QSIM assay for the compound, often giving greater sensitivity and specificity than is possible in a similar assay using electron impact (EI) ionisation. Stable isotopically-labelled analogues of compounds to be measured come closest to meeting the characteristics of ideal internal standards. These compounds behave almost identically to the analyte throughout the extraction, chromatographic separation and ionisation processes, so that the weight ratio of the added internal standard to analyte will remain unaffected by any of these operations.

This thesis is concerned with drug analysis by mass spectrometry. The detection and identification of drugs and their metabolites, often at very low levels in a complex biological matrix such as blood, can be a very difficult and demanding task. Mass spectrometry is often the only analytical method with the necessary sensitivity and specificity to unambiguously identify and then measure the analyte. The first three sections of this thesis describe the application of GCMS facilities available in the Chemistry Department at the University of Canterbury to three specific drug-related problems. The first section describes a study of morphine pharmacokinetics in patients

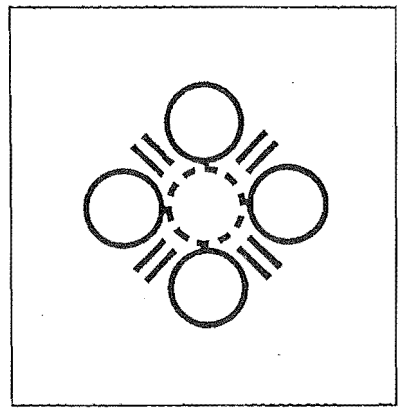
with renal failure. Plasma morphine concentrations in four patients with renal failure and in three healthy volunteers were measured using CI QSIM, and morphine pharmacokinetics in the two groups compared. The second section describes the development of a CI QSIM assay for measuring the antiparkinsonian drug benztropine in post-mortem specimens. This assay was developed for use by a nearby Government laboratory which had reported difficulties in detecting this drug. The third section describes the measurement of the atropine concentration in an aqueous extract prepared from the Solanaceous plant Datura stramonium. There is an increasing incidence of intoxication and poisoning following abuse of preparations from this plant and it was of interest to determine the level of atropine likely to be present in aqueous Datura stramonium extracts.

The fourth and final section of the thesis is concerned with drug analysis by mass spectrometry using a heated probe. There are a significant number of biochemically important compounds which are not sufficiently volatile or which are too thermally labile to be analysed using a conventional heated solids probe. Desorption chemical ionisation (DCI) and desorption electron ionisation (DEI) are two similar techniques which have recently been developed for obtaining spectra of such compounds. This section describes modifications made to a quadrupole mass spectrometer to allow it to be used with a commercially available DCI probe.

1.2 The HP5982A GCMS

The work described in this thesis was carried out using a Hewlett-Packard 5982A GCMS. This is a quadrupole instrument with an ion source that can be configured for either EI or CI operation. Reconfiguring the source physically changes the ion chamber and can be accomplished without breaking the source vacuum. The mass filter is actually a "dodecapole" because it has four pairs of tunable electrodes or "blades" between the cylindrical quadrupole rods (Figure 1). These blades modify the electric field produced by the radio frequency and direct current voltages applied to the rods so that it approximates the field which would be generated by an hyperbolic rod assembly. The mass spectrometer is pumped differentially by two high capacity oil diffusion pumps. Each diffusion pump is backed by a mechanical rotary pump. Samples are admitted to the mass spectrometer via two gas chromatographic column inlets, or through a direct insertion probe (DIP) inlet using a batch inlet probe or a heated solids probe. The CI reagent gas is admitted through the DIP inlet, or through the chromatographic column inlets when it is the GC carrier gas. Mass spectra can be recorded over the mass range 3-1000 atomic mass units, or up to four separate ions monitored using a multiple ion detector. Spectra are recorded with a light-beam oscillograph on light sensitive chart paper, and selected ion chromatograms are recorded with a conventional chart recorder connected to the multiple ion detector. A block diagram of the instrument is presented in Figure 2.

Figure 1. Cross section diagram of the HP5982A GCMS dodecapole mass filter



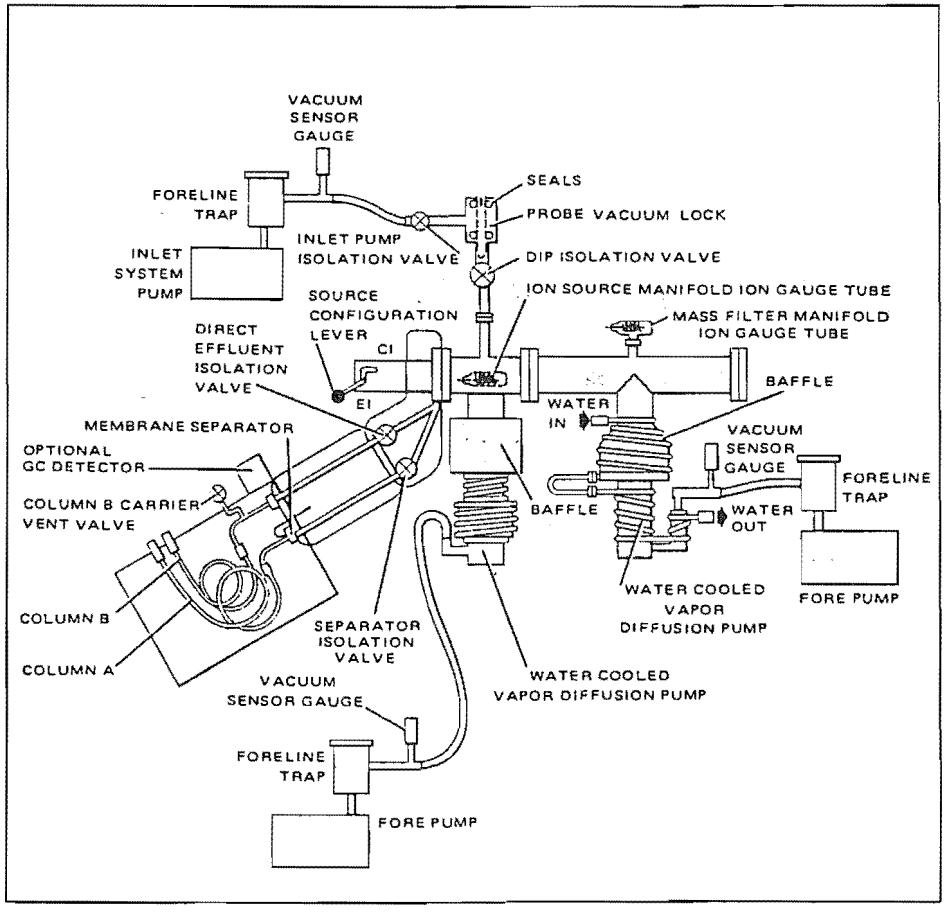


Figure 2. Block diagram of the HP5892A GCMS

Several substantial modifications were made to the HP5982A GCMS used for the work described in this thesis. These changes were largely to the mass spectrometer ion source. To aid in describing these modifications in later sections a detailed description of the original ion source is given here.

Exploded-view scale drawings of the ion source are presented in Figures 33 to 35. The source proper is housed inside a gold plated shroud (1) containing the source heater, which is controlled by a thermocouple (2), and two electron beam collimating magnets (3). Attached to the shroud is the entrance lens (4) which injects ions from the source into the quadrupole mass analyser. The EI ion chamber is a circular cavity in the ion chamber body (5) to which is attached the electron filament assembly (6) and the electron trap assembly (7). The ion chamber has four apertures, one each for the electron beam entrance, the electron beam exit, the inlet for the first GC column, and a combined DIP inlet and inlet for the second GC column. The GC column effluents are delivered to their respective inlets via glass-lined stainless steel tubes (8), and the DIP which is introduced into the source via the solids probe insertion lock is correctly positioned near the ion chamber by the DIP sleeve (9). The plunger assembly (10), mounted on a support shaft (11) and held in position by a screw (12), houses the CI ion chamber. The chamber is formed by the repeller (13), the ion exit (14) and a spacer (15). These components are electrically insulated from the plunger body by two ceramic insulators (16) and are held in position by a lock ring (17). A needle assembly (18) makes electrical contact with

both the repeller and the ion exit but is otherwise insulated from the plunger and other source parts. The spacer (15) of the plunger assembly has three apertures which are coincident with three similar apertures in the plunger body. These function as the electron entrance, the inlet for the first GC column, and the combined DIP inlet and inlet for the second GC column when the source is configured for CI. The plunger assembly can be moved into or out of the EI ion chamber by movement of the support shaft (11). This shaft passes through the end flange (19) of the ion source housing and is moved by a lever mechanism. A stainless-steel bellows maintains the seal between atmosphere and the ion source interior during plunger movement.

The two ion source configurations are depicted schematically in Figure 36. In the EI configuration the plunger is withdrawn to the left of the ion chamber body. Sample molecules are admitted to the EI ion chamber via the GC column inlet or the combined DIP and GC column inlet and ions are formed by impact with a beam of thermally emitted electrons travelling perpendicular to the mass analyser axis. The filament current is regulated to give a constant electron trap current, as is usual practice, ensuring constant electron flux in the ion chamber. The ion exit (14) of the CI ion chamber functions as an ion repeller electrode to aid extraction of positively charged ions from the source. The necessary electrical potential on this electrode is applied via the needle assembly. To change from the EI to the CI configuration the plunger is moved inside the EI ion chamber until the electron entrance and

sample inlet (both GC column, and combined DIP and GC column) apertures of both ion chambers are coincident. The needle assembly now contacts only the repeller (13) and blocks the hole in it. During CI operation sample molecules are ionised by reaction with gaseous reagent ions, formed from the reagent gas by a combination of electron impact ionisation and ion molecule collisions. Positively charged ions leave the ion chamber through the aperture in the ion exit (14). An electrical potential applied to the repeller (and thus to the complete ion chamber) via the needle assembly aids ion extraction. The CI ion chamber has no electron exit aperture because the electron beam is completely attenuated by the reagent gas. The trap current cannot therefore be used to control the filament current and so the latter is regulated to give a constant electron current to the ion chamber.

1.3 Modifications to the HP5982A GCMS

Two modifications had been made to the HP5982A GCMS by earlier users of the instrument:

(a) The original dual packed columns, membrane separator, jet separator and heated blanket interface had been removed. These had been replaced with a capillary column and a commercially available heated interface (SGE Pty, Victoria 3134, Australia). The original packed column injector had been modified for use with the capillary column.

(b) The CI reagent gas line had been connected to the unused mass spectrometer GC inlet. This allowed the reagent gas to be introduced into the source without it first

passing through the GC column when CI spectra of probe samples were being recorded.

When this work began considerable difficulty was experienced with two needle valves in the SGE heated interface between the gas chromatograph and the mass spectrometer. These valves, one for isolating the mass spectrometer from the gas chromatograph and the other for controlling the gas flow passing through the interface, were found to leak. Further problems were encountered with cracks developing in the glass-lined metal tube (8) inside the source which delivers the GC column effluent to the EI and CI ion chambers (in the modified instrument one glass lined tube carries the CI reagent gas). These cracks exposed the column effluent to a bare metal surface, causing a dramatic loss in sensitivity when measuring trace amounts of analyte. In an attempt to solve these problems the capillary column was fed directly into the source (bypassing the two troublesome valves) and through the glass-lined tube to within 1 cm of the ion chamber. Unfortunately, the sharp bends in the glass-lined tube placed considerable strain on the column, causing it to break frequently. This also occurred when the glass-lined tube was replaced with an all-glass tube of the same dimensions but having bends of a slightly larger radius. The following modifications to the source were therefore made. A 6 mm diameter hole was cut in the side of the source mounting ring (20) and similar holes were cut in the source sealing shoe shim springs (21). The hole in the ion chamber body (5) and the hole in the source sealing shoe (22), both for receiving the original glass-lined tube carrying the column effluent, were

counter-bored to a diameter of 3.97 mm. A short length of 4 mm Teflon tubing was passed down through the ion chamber body supporting cylinder (23) and through the new holes in the source mounting ring and in the shim springs. One end of the Teflon tubing was pushed tightly into the enlarged hole in the ion chamber body and the other end was pushed tightly into the enlarged hole in the source sealing shoe. The unused hole in the ion chamber body (which in the modified instrument was the entry port for the CI reagent gas) was blocked off with a short length of stainless steel rod. The ion source was then reassembled and returned to the instrument. The external inlet for the second column was capped, and the capillary column was fed into the remaining inlet, through the Teflon tubing, which acted as a guide, and directly into the ion source. A T-connector at the external inlet allowed the CI reagent gas to enter at this point and flow through the Teflon tubing (and around the column) and into the ion chamber.

In any assay using QSIM it is important to ensure that the mass spectrometer is set to monitor the correct m/z values, and that there is no drift from these values once they are set. Each channel of the HP5982A's multiple ion detector (MID) supplies a tuning voltage of 0 to +10 volts to control the mass spectrometer over the mass range 0 to 1000 atomic mass units. The four (or less) masses to be monitored are selected by adjusting four ten-turn potentiometers on the MID's front panel. The selected mass is displayed on a digital voltmeter (DVM). In this work, setting the required masses on the MID as originally supplied proved difficult. The large mass range covered by

each turn of the mass selection potentiometers meant that considerable care was required in adjusting these controls. Also, the last digit on the DVM displaying the selected masses was difficult to read and changed frequently, although numerous checks of the MID output voltages showed them to be stable. To solve these difficulties a new DVM was attached to the MID, and two resistors were inserted in the mass selection potentiometer circuit to reduce the mass range covered by the potentiometers. The details of this modification are described in Appendix A.

An OCI-3 on-column injector (SGE Pty.) was fitted to the HP5982A's gas chromatograph and used for much of the work described in this thesis. The fitting of the injector and its associated gas lines (including a flow controller) was straightforward and will not be described.

CHAPTER 2

INTRODUCTION

2.1 Morphine

Morphine, an alkaloid isolated from opium, is generally considered to be the most valuable of all pain relieving drugs. It is widely used to relieve moderate to severe pain associated with acute and chronic disorders, to provide analgesia during diagnostic and orthopaedic procedures and as a preoperative medication before surgery.

Morphine is variably absorbed from the gastrointestinal tract after oral ingestion and is thought to have a high first-pass hepatic extraction ratio (see note 1, Appendix B) so it is usually administered subcutaneously (S.C.), intramuscularly (I.M.) or intravenously (I.V.). After S.C. or I.M. injection absorption into the bloodstream is rapid and the drug is distributed throughout the body, but mainly in the kidneys, liver, lungs and spleen, with lower concentrations in the muscles. The brain is the main site of action of morphine but relatively little of an administered dose penetrates the blood-brain barrier, the mechanism which prevents many substances in the blood from entering the central nervous system[1].

The dosage of morphine that must be administered to a patient in pain to provide analgesia depends on the degree of analgesia required, the patient's response and the route by which the drug is given. Morphine given parenterally in a dose of 0.1-0.15 mg/kg body weight will achieve a plasma concentration of about 0.05 $\mu g\ mL^{-1}$. This is the plasma concentration of morphine required to relieve moderate to severe pain and peak analgesia occurs within 20-90 minutes.

The excretion of morphine from the body is mainly via the urine, and is preceded by extensive metabolism to derivatives which are excreted more rapidly than the parent drug. The main metabolic pathway is conjugation with glucuronic acid to form morphine-3-glucuronide. Minor morphine metabolites which have been identified in human morphine addicts and/or non-addict patients are morphine-6-glucuronide in plasma and urine, and morphine-3,6-diglucuronide, morphine-3-ethereal sulphate, normorphine, normorphine-6-glucuronide and (tentatively) normorphine-3-glucuronide in urine. In healthy subjects (i.e. non-addict patients) with normal renal function 3-10% of an administered dose is excreted unchanged in the urine, about 70% as morphine-3-glucuronide and about 5% as normorphine. About 90% of total urinary excretion occurs within 24 hours. A small amount of a morphine dose is excreted unchanged into the faeces, largely in the bile[2].

2.2 Morphine Disposition During Renal Failure

Patients with renal failure often appear to be more sensitive to the effects of morphine than patients with normal renal function[3-8]. Adverse drug reactions by renal failure patients to a therapeutic morphine dose include an unexpected degree of respiratory depression and a prolonged duration of drug action. The implication is that morphine distribution and elimination (disposition) is altered in some way by renal failure, in spite of the traditional belief that the liver is the main site of morphine metabolism[9].

Disturbances of drug-protein binding during renal failure have been suggested as a cause, at least in part, for increased sensitivity to morphine[10]. Most drugs, including morphine[11], normally bind reversibly to some extent to plasma proteins and changes to this binding, caused by altered concentrations or an altered binding capacity of proteins, can lead to unusually high or low plasma levels of unbound and active drug. Morphine binding in renal failure has been shown to depend on the concentration of total serum proteins and albumin[10] and both of these concentrations are often diminished by renal failure[10].

Morphine disposition during impaired renal function has been studied by comparing the pharmacokinetics of I.V. morphine in healthy volunteers and in patients with end-stage renal failure[12]. Plasma unchanged morphine was measured using high pressure liquid chromatography (HPLC). The rates of morphine elimination in the two groups were similar but morphine concentrations were higher in the patients than in the volunteers for the first 15 min after administration, implicating high early morphine concentrations in increased sensitivity to the drug. It was suggested that morphine-3-glucuronide, a major morphine metabolite[9,13] that can produce analgesia and respiratory depression in dogs[14] but which is thought to be inactive in humans[15], probably accumulates in the plasma of anuric patients (those with no kidney function).

The pharmacokinetics of morphine in patients undergoing renal transplantation and in control subjects have also been

compared[16]. Using a "specific" radioimmunoassay (RIA) method[17] to measure unchanged plasma morphine, the transplant patients were shown not to eliminate the drug from plasma until the transplant kidney began to function, at which point there was an abrupt change to the same elimination half-life as the controls. This suggested that patients with renal failure would accumulate morphine, rather than eliminate the drug normally as had been previously reported.

The group that demonstrated altered morphine pharmacokinetics in renal transplantation has recently reported unchanged morphine accumulation and reduced morphine clearance (volume of plasma cleared in unit time) in patients with renal failure[8,18]. The clearance of morphine was related linearly to creatinine clearance[18]. (Creatinine clearance is considered a good indicator of glomerular filtration rate and thus of renal function[19]). There has also been another report, by a different group, of morphine accumulation and reduced clearance during renal failure[20]. In all studies reporting sustained high plasma morphine concentrations the analytical method used to measure morphine was RIA.

The finding that morphine elimination began only after the recovery of renal function during renal transplantation has been interpreted as demonstrating an important role for the kidney in unchanged morphine elimination, and led, in part, to the suggestion that the kidney, and not the liver, is the main site of morphine metabolism. This "renal hypothesis" or "renal glucuronidation theory", proposed by McQuay and Moore[6], was supported by the reports

of unchanged morphine accumulation and reduced clearance during renal failure, and by other evidence that hepatic morphine metabolism may not be as important as is widely believed. Patients with severe hepatic cirrhosis have been found to tolerate morphine normally (although intolerance has also been reported[21]) and to have morphine clearances similar to those in volunteers with normal liver function[22]. Drugs metabolised by the liver would be expected to have reduced clearance during cirrhosis and this has been found for pethidine and pentazocine[23]. Coadministration with morphine of drugs that lower liver blood flow would also be expected to reduce morphine clearance if morphine metabolism were primarily hepatic but two such drugs, cimetidine[24] and propranolol[25], both appear to have no effect on clearance. Biochemical evidence suggests that a renal mechanism for morphine metabolism in humans may exist; rabbit kidney tubule is able to accumulate morphine and to metabolise the drug to its glucuronides[26].

All in all, there is considerable confusion regarding the disposition of morphine in renal failure and the role of the kidney in morphine metabolism. The studies suggesting that unchanged morphine elimination is impaired in renal failure, and which support the renal hypothesis, used an RIA method to determine plasma unchanged morphine concentrations. The validity of this assay has recently been questioned[27,28]. An RIA using a similar antiserum (from the same animal) has been shown to overestimate unchanged morphine when morphine:morphine-3-glucuronide concentration ratios are greater than 1:10[27]. Morphine-3-glucuronide is present in chronically treated

patients at a concentration up to twenty-five times that of morphine[29] and this may have contributed significantly to the unchanged morphine concentration being measured. Another morphine metabolite, morphine-6-glucuronide[29] may also have cross-reacted with the morphine antisera[28].

2.3 This Work

Papaveretum (Omnopon®, Roche) is a standardised mixture of four opium alkaloid hydrochlorides. It contains the equivalent of anhydrous morphine 47.5 to 52.5%, anhydrous codeine 2.5 to 5%, noscapine 16 to 22%, and papaverine 2.5 to 7%[30]. Papaveretum is widely considered superior to morphine in providing analgesia and sedation[31,32], and is the most commonly used opoid in many New Zealand hospitals[33].

This section of the thesis describes a preliminary pharmacokinetic study of unchanged morphine in four patients with renal failure (one anephric) and three healthy volunteers after the intramuscular administration of papaveretum. The pharmacokinetics of morphine glucuronides in two of the patients were also studied. Unchanged morphine concentrations in serum samples were determined using GCMS with ammonia chemical ionisation mass spectrometry and quantitative selected ion monitoring. Morphine glucuronide concentrations were determined as the difference between morphine concentrations measured before and after liberation of the conjugates by enzyme hydrolysis. The study was prompted by the observation that papaveretum

occasionally has an excessive effect in renal failure patients[33] and the disposition of morphine in such patients is still uncertain.

RESULTS AND DISCUSSION

2.4 Analytical Methods

2.4.1 Discussion of Preparative Procedures

(a) N-Trideuteriomethylnormorphine

N-Trideuteriomethylnormorphine ([$^2H_3$]-morphine, D_3-morphine) was used as the internal standard for the quantitation of morphine in plasma and serum by GCMS. This compound is a stable isotope analogue of morphine and provides a number of advantages over other types of internal standard (see Section 1.1).

D_3-morphine was already available[34]. To ensure the compound's purity it was recrystallised from methanol/water and then sublimed under vacuum. Mass spectral analysis showed the level of [$^1H_3$]-morphine (D_0-morphine) contained in the purified D_3-morphine to be less than 1%.

(b) Morphine-(3β-D-glucopyranosyl)-uronate

Morphine-(3β-D-glucopyranosyl)-uronate (morphine-3-glucuronide) was prepared from morphine and methyl-(tri-O-acetyl-α-D-glucopyranosyl bromide)-uronate using the method of Berrang[35] (Figure 3). The crude product was purified by repeated recrystallisation from methanol. Methyl(tri-O-acetyl)-α-D-glucopyranosyl bromide)-uronate was formed by the acid catalysed acetylation of glucuronolactone to give methyl(tetra-O-acetyl-β-D-glucopyranuronate)[36] which was then treated with hydrobromic acid[36].

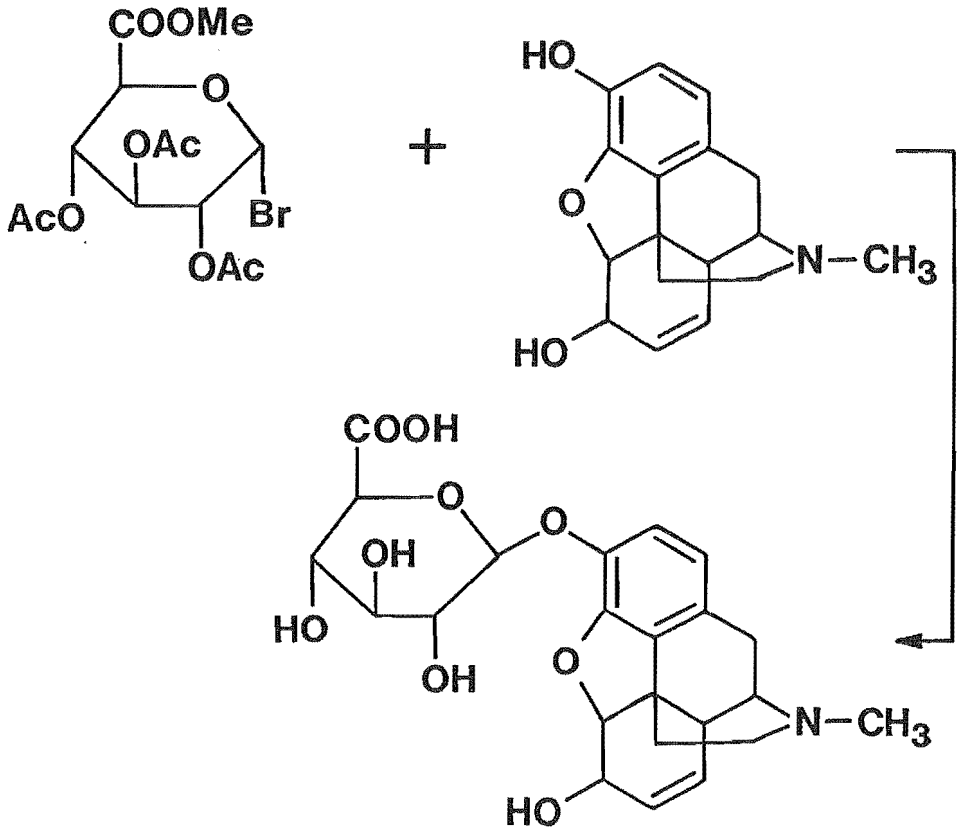


Figure.3. Formation of morphine - 3 - glucuronide

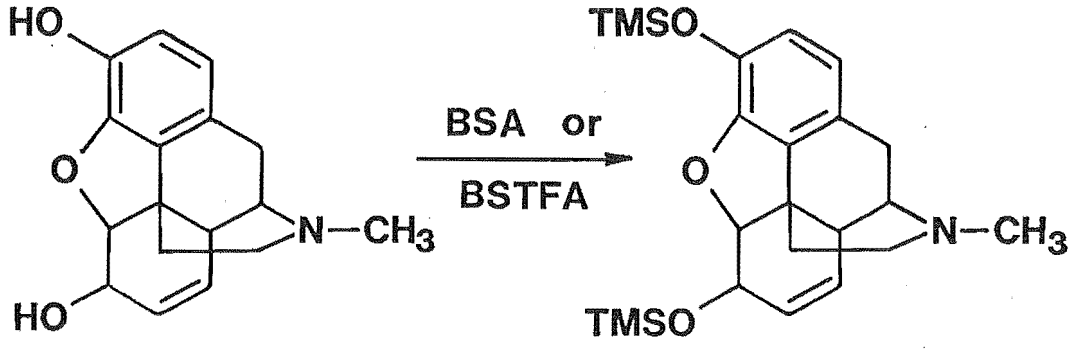


Figure.4. Derivatisation reaction for morphine

(TMS=$Si(CH_3)_3$)

2.4.2 Derivative Formation

The gas chromatographic behaviour of morphine base is improved by derivatisation. This procedure increases the drug's volatility, and prevents its phenolic and hydroxyl groups interacting with the GC column to cause adsorption and tailing. Trimethylsilylmorphine derivatives were formed (Figure 4), prior to GCMS analysis, by reacting the dried residue of serum or plasma extracts containing morphine with N,O-bis-(trimethylsilyl)-acetamide (BSA) or N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) for 1 h at 60°C. GC analysis showed that derivatisation was completed in this time.

2.4.3 Extraction Method for Morphine

Morphine was extracted from plasma and serum by liquid-liquid extraction. The procedure was based on previously reported methods[37,38] and is fully described in the experimental section. All glassware was silanised to avoid loss of analyte and internal standard by adsorption onto glass surfaces.

Plasma and serum were extracted as 1 mL aliquots in 10 mL glass tubes with ground glass stoppers. The aliquots were spiked with internal standard, thoroughly mixed, and allowed to stand overnight to equilibrate (samples being analysed for total morphine were incubated with β-glucuronidase before extraction). Proteins were precipitated with acetone, and the acidified aqueous phase washed with benzene to remove acidic and neutral endogeneous material. (Washing the aqueous phase with toluene, a solvent which is less toxic than benzene, reduced the

recovery of morphine). The aqueous phase was then made alkaline, and morphine extracted with dichloroethane containing 30% (v/v) isopropyl alcohol. Morphine is an amphoteric drug and most efficiently extracted into an organic solvent from aqueous solution at a pH near its isoelectric point of 8.9. This pH was attained using sodium hydroxide and a borate buffer. The dichloroethane-isopropyl alcohol was selected after evaluating six solvents or solvent mixtures by GC with flame ionisation detection, and ammonia CI GCMS for convenience, efficiency of morphine extraction, and ability to discriminate against unwanted endogeneous material (Table 1). After the extraction procedure was completed, the organic solvent was reduced in volume, transferred to a Reacti-vial and evaporated to dryness. The dried residue containing morphine was derivatised just prior to GCMS analysis.

The recovery of morphine from plasma, using the extraction procedure finally adopted, was determined at morphine concentrations of 50 ng mL^{-1} and 200 ng mL^{-1} by ammonia CI GCMS and the results are shown in Table 2. No morphine was detected in plasma spiked with morphine-3-glucuronide at 200 ng mL^{-1}.

Solid phase extraction techniques offer a number of advantages over liquid-liquid extractions[39,40], and an attempt was made to extract morphine from plasma using Sep-pak C_{18} reverse phase cartridges (Waters Assoc.). Plasma spiked with morphine and morphine-3-glucoronide was aspirated through prewashed (methanol, water, buffer) cartridges and the morphine eluted with methanol. A good recovery of morphine was obtained but morphine-3-glucuronide

Solvent	% morphine recovery from plasma* (n=5)	Notes
n-butyl chloride	15-25%	very clean extracts
Hexane	55-70%	Problems with emulsions
Ethyl acetate	30-38%	High level of co-extracted material
Toluene-butanol	60-70%	Difficult to evaporate
Benzene	58-62%	Toxic

Table 1 . Solvents evaluated for extraction of morphine

*Plasma morphine concentration 50 ng mL^{-1}.

Plasma morphine concn.	% Recovery (mean of 5)	Range
50 ng mL^{-1}	76	73-81
200 ng mL^{-1}	83	79-87

Table 2 . Recovery of morphine from plasma using dichloroethane containing 30% (v/v) isopropyl alcohol.

was also detected in the extracts. Elution of the conjugate could be prevented by washing the cartridge with water but this lowered the recovery of morphine to an unacceptable level.

2.4.4 Hydrolysis of Conjugated Morphine

Conjugated morphine in plasma and serum was hydrolysed using the enzyme β-glucuronidase (Sigma Type H-1 from *Helix pomatia*). This enzyme also has some sulphatase activity. Plasma and serum samples were hydrolysed as 1 mL aliquots in the same glass tubes that were used for extracting unconjugated morphine. The samples were buffered to pH 5 and hydrolysed with the enzyme in a heated water bath at 37° for 24 h, in accordance with the enzyme manufacturer's instructions. The minimum quantity of β-glucuronidase (measured in Fishman Units) required to give the maximal yield of liberated drug was determined by the following procedure: Plasma containing morphine-3-glucuronide at a concentration of 5 μg mL^{-1} was prepared by adding a known mass of the conjugate to drug-free plasma. Aliquots of this plasma were spiked with D_3-morphine and these samples were then hydrolysed with 50, 100, 500, 1000, 2000, 3000, 4000 or 5000 Fishman Units of enzyme using the method which has just been described. After hydrolysis the samples were extracted and analysed using the method for unconjugated morphine. The yield of liberated drug, determined by measuring the ratio of D_0- and D_3-morphine, was found to increase and maximise at 2000 Fishman Units. The exact yield of liberated morphine was

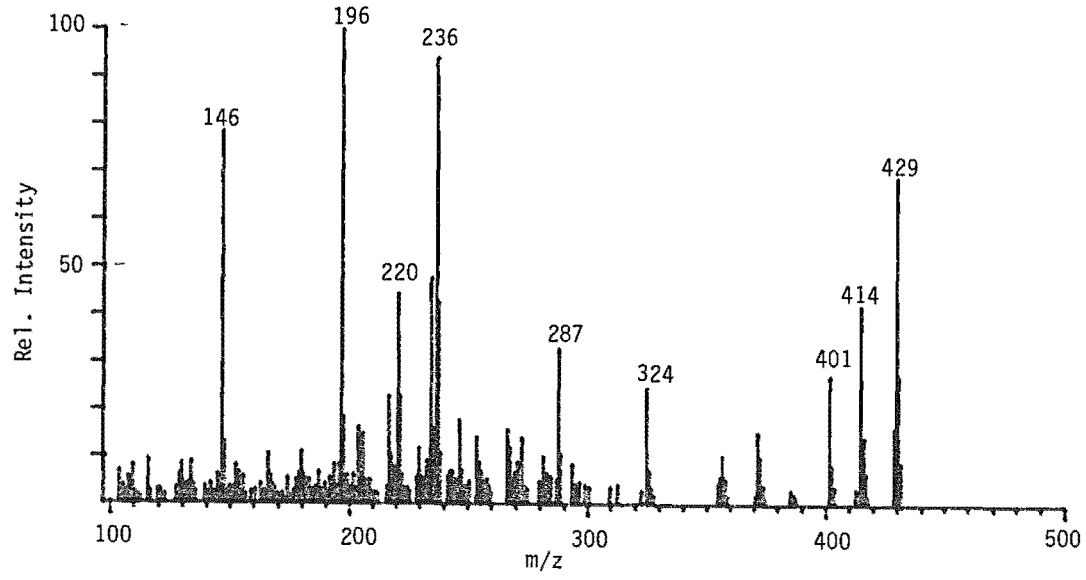
not determined as no calibration graph was constructed, but this was estimated to be about 80% of available morphine.

2.4.5 Mass Spectrometry of Derivatised Morphine

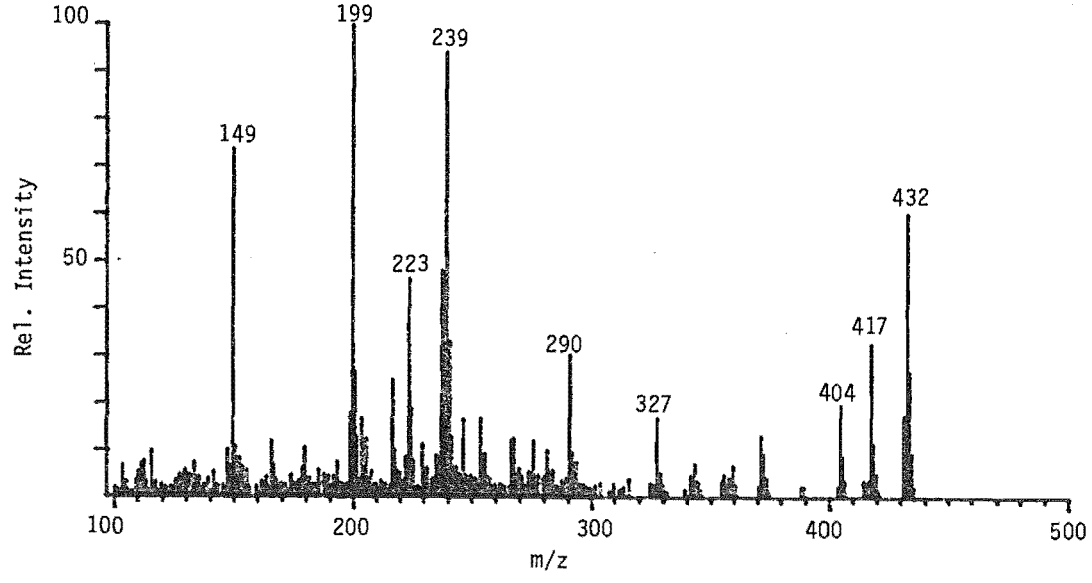
The EI mass spectra, and methane, isobutane and ammonia CI mass spectra, for TMS_2-D_0-morphine and TMS_2-D_3-morphine were obtained and are shown in Figures 5 and 6. The EI spectra show molecular ion peaks (M^+) (m/z 429 and 432), and extensive fragmentation with base peaks m/z 196 and 199 respectively. The CI spectra show protonated molecular ion peaks (MH^+) (m/z 430 and 433), and much less fragmentation. The base peak with ammonia reagent gas is MH^+ but with methane and isobutane it is a fragment ion. Both compounds show less fragmentation with ammonia than with methane or isobutane. All the CI spectra show peaks corresponding to the electrophilic attachment of reagent gas ion, with a large fraction of the total ion current carried by MH^+ - methane (5%); isobutane (17%) and ammonia (32%). This suggested that monitoring MH^+ under CI, particularly with ammonia reagent gas, might provide high sensitivity for the quantitation of morphine. Furthermore, the CI spectra all show sufficient fragmentation to allow high specificity to be attained by monitoring more than one ion.

The sensitivities of an assay based on monitoring ions generated by either EI or CI, however, cannot be determined from normalised mass spectra[41,42] since the ionisation cross section varies widely from EI to CI, and from one reagent gas to another. A comparison of sensitivities was made experimentally by injecting equal amounts of TMS_2-morphine into the GCMS while the mass spectrometer was monitoring m/z

Figure 5. EI mass spectra of TMS_2-D_0- and TMS_2-D_3-morphine.

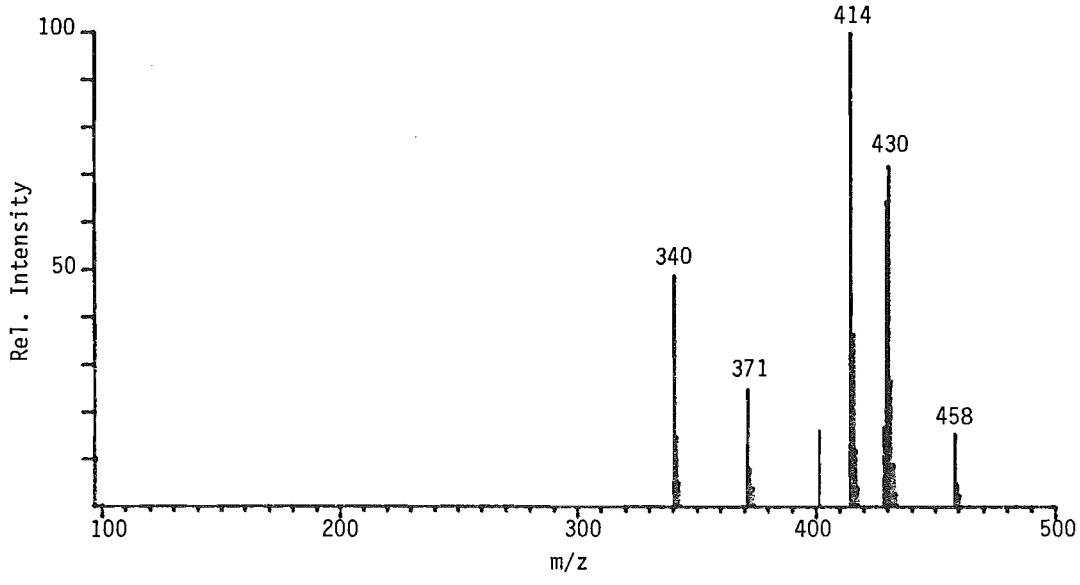


(a) EI mass spectrum of TMS_2-D_0-morphine

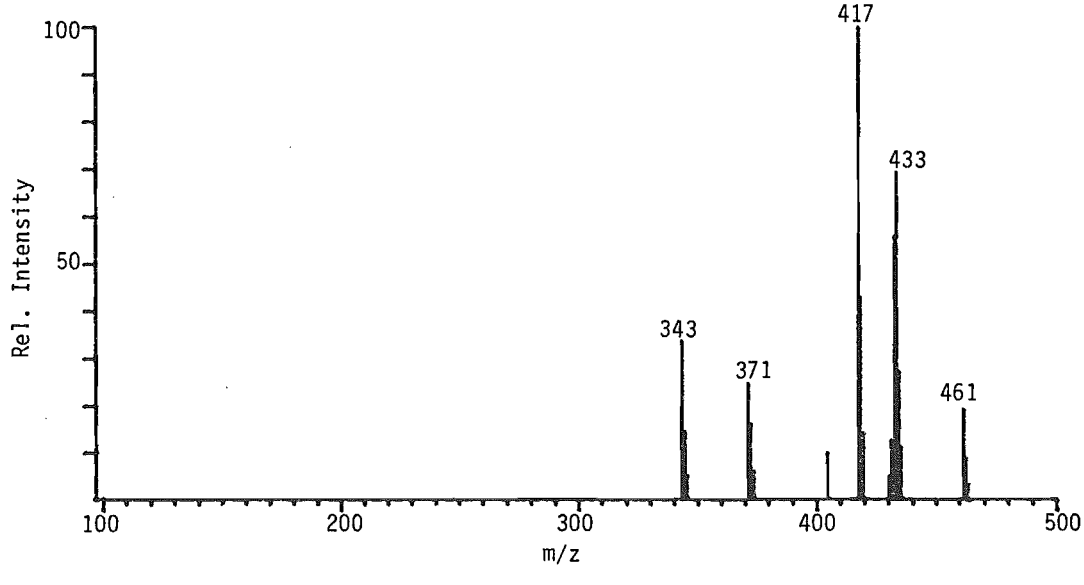


(b) EI mass spectrum of TMS_2-D_3-morphine

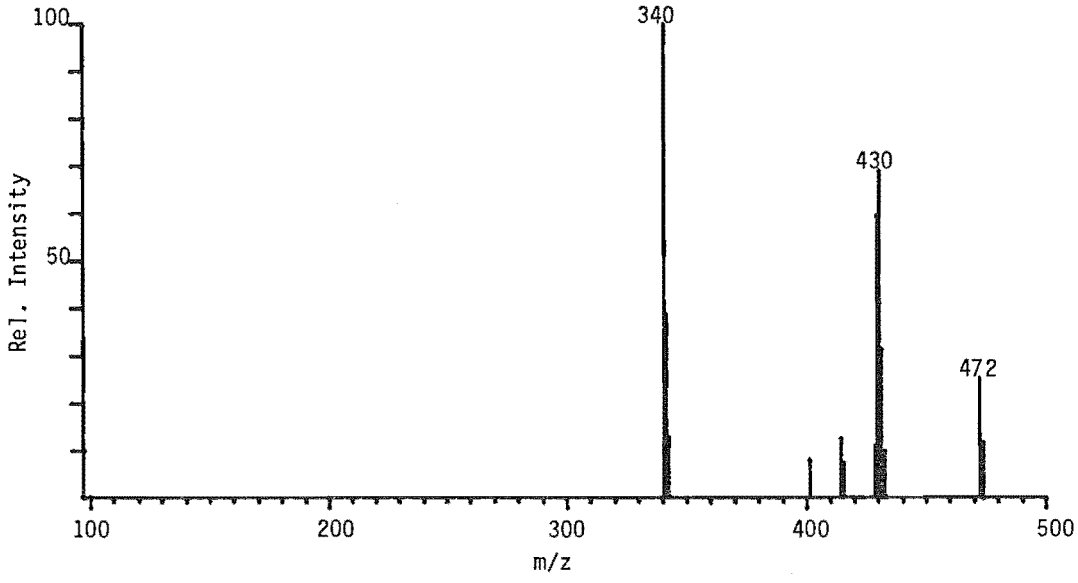
Figure 6. CI mass spectra of TMS_2-D_0- and TMS_2-D_3-morphine using CH_4, C_4H_{10} and NH_3 as reagent gases.



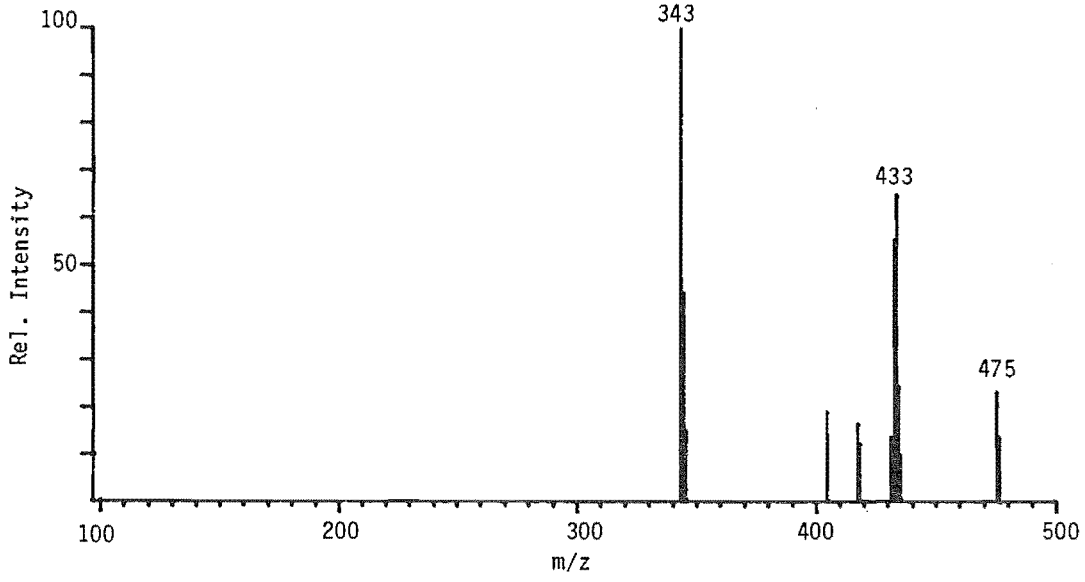
(a) CI(CH_4) mass spectrum of TMS_2-D_0-morphine



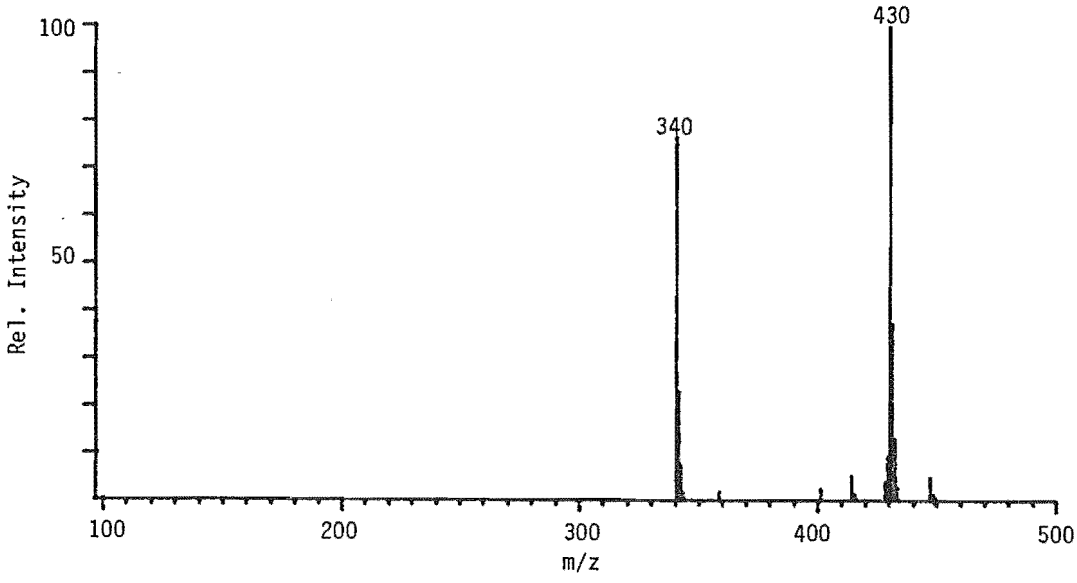
(b) CI(CH_4) mass spectrum of TMS_2-D_3-morphine



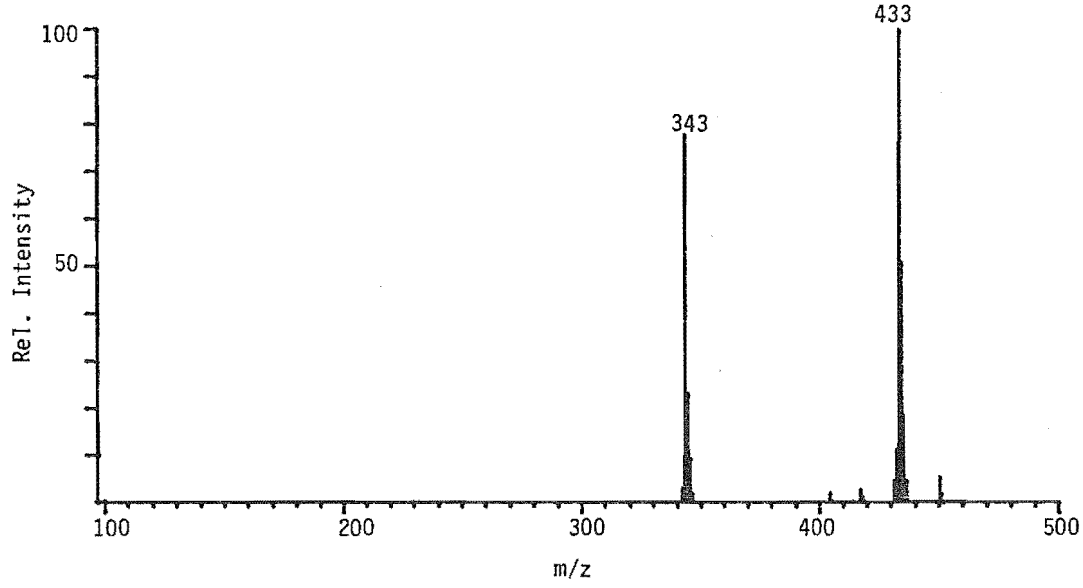
(c) CI(C_4H_{10}) mass spectrum of TMS_2-D_0-morphine



(d) CI(C_4H_{10}) mass spectrum of TMS_2-D_3-morphine



(e) CI(NH_3) mass spectrum of TMS_2-D_0-morphine



(f) CI(NH_3) mass spectrum of TMS_2-D_3-morphine

429 using EI and then m/z 430 using CI for all three reagent gases. (The MS was retuned for maximum sensitivity in each case). Ion intensities were determined and compared by measuring peak heights on a chart recorder. The CI response for MH^+ with ammonia was about twice that obtained with methane or isobutane, which in turn gave about the same response as the EI molecular ion. CI sensitivities were also compared for the base peak of TMS_2-morphine with methane (m/z 414), isobutane (m/z 340) and ammonia (m/z 430), and again the response was greatest with ammonia. Based on these results, ammonia CI was selected for the quantitation of morphine in plasma and serum extracts.

Ammonia CI was found to offer another advantage over EI, in addition to a lower detection limit. An ammonia plasma generally protonates only those compounds containing either a basic nitrogen or certain carbonyl functions[43]. At least four compounds with retention times close to that of morphine were detected in derivatised plasma and serum extracts when monitoring M^+ at high gain by EI GCMS. Only one other compound was detected when monitoring MH^+ in CI with ammonia reagent gas.

2.4.6 Instrument Preparation

A serious limitation to the sensitivity obtainable when determining trace levels of an organic compound by GCMS is imposed by adsorption or absorption of the analyte and the internal standard onto the mass spectrometer inlet system. There have been many reports of this problem,[42,44] which is solved, in part, by saturating the inlet system with the compounds being determined. In this work the GC column and

the ion source were saturated with TMS_2-D_0-morphine and TMS_2-D_3-morphine before beginning analysis of the serum extracts, and following any dismantling, cleaning or change to the ion source or transfer line. Saturation was achieved by 20-30 injections of 100 ng of the two derivatives, and was followed by repeated injections of methanol until no mass spectrometer response was observed under conditions of high gain at m/z 430 and 433.

The mass spectrometer's sensitivity was found to vary significantly from day to day. The instrument was therefore retuned on each occasion plasma or serum extracts were analysed. Reference compounds used for tuning were introduced into the ion source using a conventional direct-insertion probe. The mass spectrometer was tuned for ammonia CI operation by maximising the m/z 490 peak of chloropentaiodobenzene[45] and using this peak and the ^{37}Cl isotope at m/z 492 for resolution adjustment. Final tuning was optimised using a 1:1 mixture of TMS_2-D_0-morphine and TMS_2-D_3-morphine. After adjusting the various ion-source and ion-focussing electrode potentials, the multiple ion detector (MID) control unit was used to set the mass spectrometer to monitor m/z 433, m/z 430 and m/z 340. The exact m/z monitored, which may be slightly different from these values because of mass defect and instrument calibration error, was set by scanning across each peak and setting the MID mass scale control to give maximum ion current, as measured by pen deflection on the MID chart recorder.

2.4.7 Quantitative GCMS Analysis

Quantitation of morphine was based on the MH^+ ion intensities from TMS_2-D_0-morphine (m/z 430) and TMS_2-D_3-morphine (m/z 433). The resulting ion intensity ratio (intensity m/z 430/intensity m/z 433) was converted to a D_0/D_3 mass ratio using a calibration graph. Ion intensities were determined by measuring peak heights on a chart recorder. In order to reduce the measuring error the mass spectrometer sensitivity was adjusted to bring the peaks to as close as possible to full-scale deflection. Figure 7 shows the response at m/z 430 and 433 for an injection of a derivatised extract from plasma that contained D_0- and D_3-morphine, and for a derivatised extract from blank plasma. A set of initial calibration graphs was prepared to establish that the relationship between the mass spectrometer ratios and mass ratios was linear, and to define the errors in the analysis. The calibration graphs were constructed using extracted and derivatised plasma samples that contained D_0- and D_3-morphine in the ratios 0.25, 0.50, 1.0 and 2.0 D_0:D_3. The preparation of the standard mixtures of D_0- and D_3-morphine used to spike the plasma samples is discussed in the experimental section. Two calibration graphs, one at a total $D_0 + D_3$ concentration of 60 ng mL^{-1} of plasma and the other at 400 ng mL^{-1}, were obtained prior to beginning the pharmacokinetic study. These graphs are shown in Figures 8 and 9, and the ion intensity ratios and statistical analysis are given in Tables 3 and 4. A weighted linear least-squares procedure[46] (see Appendix C) was used to find the regression line for both calibration graphs. The absolute standard

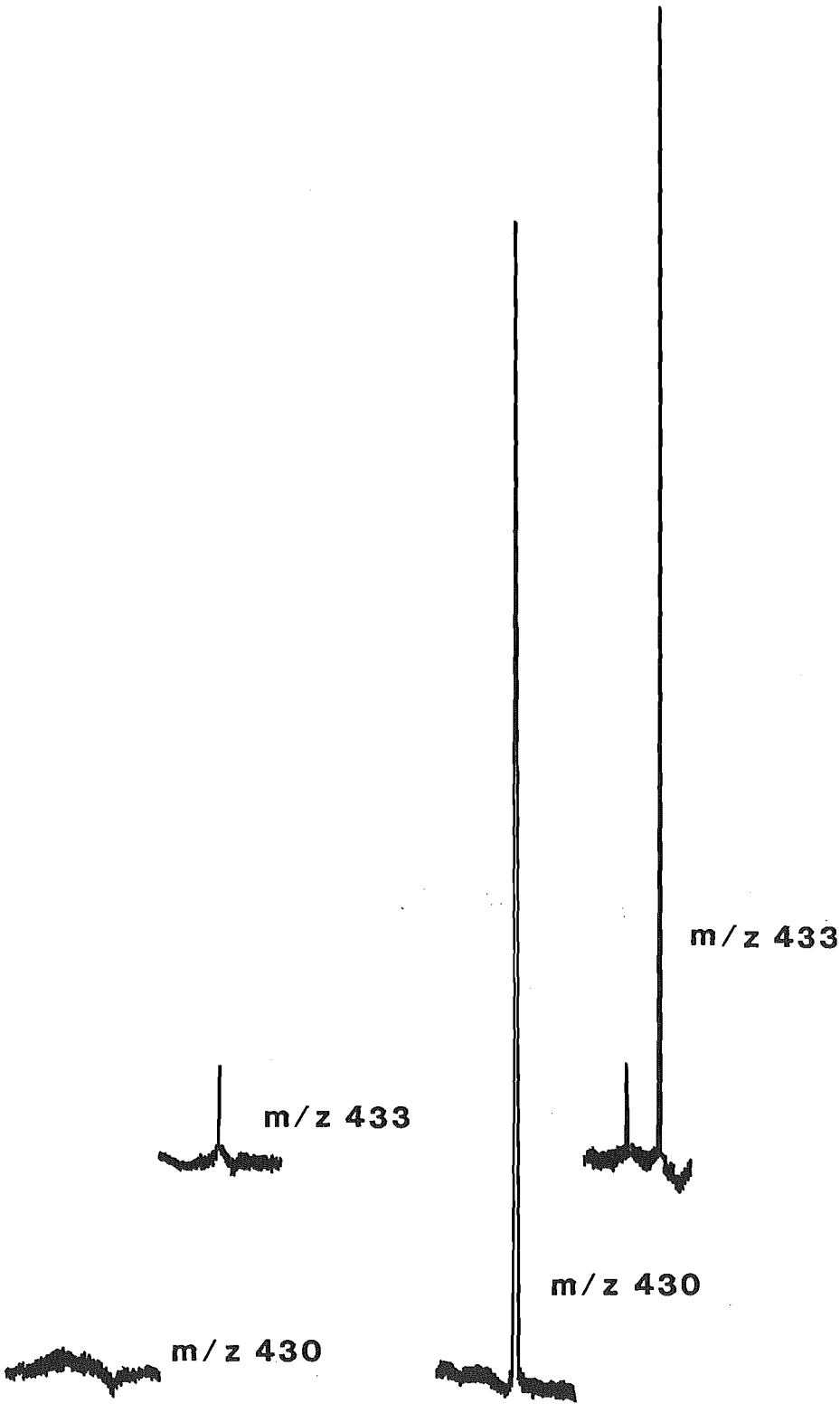


Figure 7. Response at m/z 430 and 433 near the retention time for TMS_2-morphine for (a) an extract from blank plasma, and (b) an extract from plasma doped with $D_0 + D_3 = 60$ ng mL^{-1} morphine.

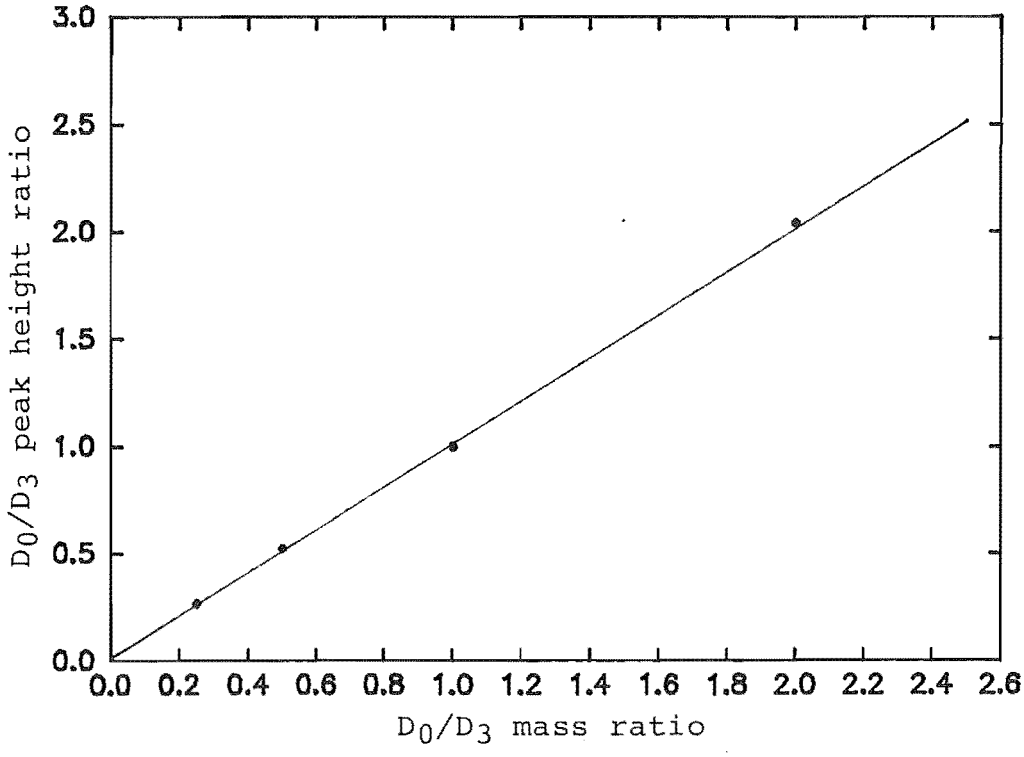


Figure 8. Calibration graph for morphine in plasma

(D_0 + D_3 = 60 ng mL^{-1}, ● = mean of five measurements)

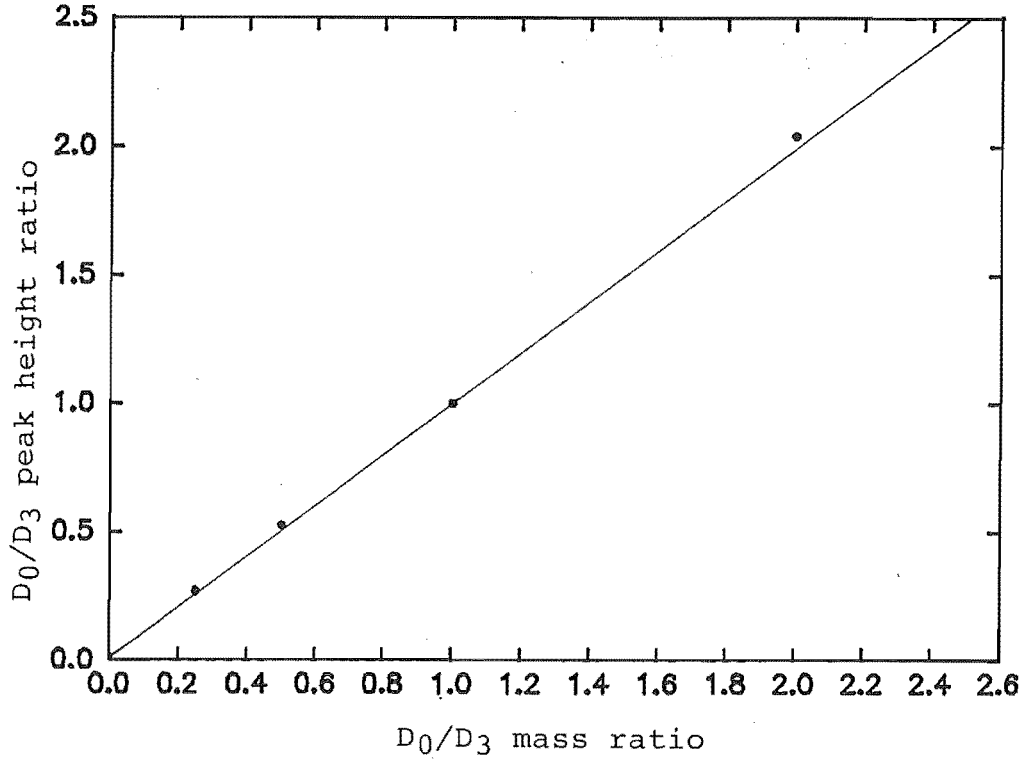


Figure 9. Calibration graph for morphine in plasma

(D_0 + D_3 = 400 ng mL^{-1}, ● = mean of three measurements)

TABLE 3 . CALIBRATION DATA AND REGRESSION ANALYSIS STATISTICS

Concn. in plasma ($D_0 + D_3$)	D_0/D_3 ratio	n	$\bar{x}$	s
60 ng mL^{-1}	0.25	5	0.267	0.02907
"	0.50	"	0.524	0.04904
"	1.00	"	0.999	0.07738
"	2.00	"	2.040	0.13148

Correlation coefficient (r)	Gradient	% of explained variation $100\ r^2$	Parameters for least squares line $y = a + bx$	Theoretical value of r at 99.9%
0.999	1.00	99.9	$y = 0.0167 + 1.00x$	0.6787

TABLE 4. CALIBRATION DATA AND REGRESSION ANALYSIS STATISTICS

Concn. in plasma ($D_0 + D_3$)	D_0/D_3 ratio	n	$\bar{x}$	s
400 ng mL^{-1}	0.25	3	0.263	0.00916
"	0.50	"	0.485	0.2214
"	1.00	"	0.976	0.04725
"	2.00	"	2.09	0.05567

Correlation coefficient (r)	Gradient	% of explained variation 100 r^2	Parameters for least squares line $y = a + bx$	Theoretical value of r at 99.9%
0.998	0.992	99.6	$y = 0.0101 + 0.992x$	0.8233

deviation in the measured intensity ratios was found to decrease as the D_0/D_3 ratio decreases, causing the smaller isotope ratios to be defined with greater precision. Weighting the regression line ensures that it is less sensitive to the large absolute errors at the upper extreme of the calibration and forces it to pass close to the more precisely defined points near the origin. The weighting factor used at each point was the inverse of the variance in the measured intensity ratios[41,42,46].

The regression analyses were carried out by treating the mass ratios from the standard plasma samples as the independent variable and the measured ion intensity ratios as the dependent variable. This approach, which is sometimes used incorrectly[41], assumes that the variance in mass ratios is less than the variance in the ion-intensity ratios[46]. It was adopted in this work because, although the 60 ng mL^{-1} and 400 ng mL^{-1} calibration data were obtained from extracted plasma samples prepared using two independent sets of standard D_0- and D_3-morphine mixtures, (one set for each level) a statistical analysis showed the two regression lines to be indistinguishable.

Plasma samples containing D_0- and D_3- morphine in the ratio 4:1, were excluded as calibration standards. Two such samples were found consistently to give points below the least-squares line on the 60 ng mL^{-1} calibration graph. This deviation was attributed to the (MH+3) peak ($^m/z$ 433) of TMS_2-D_0-morphine contributing significantly to the intensity of the MH^+ ion of TMS_2-D_3-morphine (m/z 433) in a mixture with a large proportion of D_0-morphine. Curvature in a calibration graph can also be caused by isotopic

impurity of the labelled compound. In the 1:4 D0:D_3 case, TMS_2-D_3-morphine will show a low intensity ion at (MH-3) due to the presence of a small amount of unlabelled TMS_2-D_0-morphine. The degree of non-linearity introduced by this impurity was negligible, as shown by r values of better than 0.99 for both the 60 ng mL^{-1} and 400 ng mL^{-1} calibration graphs.

The accuracy of the procedure for determining morphine in plasma was assessed using a single doped plasma sample containing morphine at a concentration of 30 ng mL^{-1}. Five 1 mL aliquots of this plasma were doped with D_3-morphine so that D_0:D_3 = 1, and another five 1 mL aliquots were doped with D_3-morphine so that D_0:D_3 = 0.5. All ten samples were extracted and analysed using the procedures which have been described. Measured morphine concentrations were 29.3 ± 1.4 ng mL^{-1} when D_0:D_3=1 and 28.4 ± 1.7 ng mL^{-1} when D_0:D_3=0.5.

2.5 Patient Study

2.5.1 Patients and Healthy Volunteers

The four patients were men with renal failure, one (4, Table 5) being anephric (without kidneys). All four were on maintenance haemodialysis (see note 2, Appendix B). The important characteristics and laboratory data for each patient were supplied by medical staff at Christchurch Hospital and are given in Table 5.

All patients had normal serum concentrations of the enzyme aspartate aminotransferase (AST), indicating that they were free of liver disease. The concentration of this

Table 5 Important characteristics and laboratory data for the renal failure patients.

Patient	Age (years)	Weight (kg)	Months on dialysis	Cause of renal failure	Other medical problems	Drugs[d]	Albumin[a] (gL^{-1})	AST[b] IU/L	Haemoglobin[c] (gL^{-1})
1	60	80	2	Polycystic kidney disease	-	-	41	11	84
2	54	65	1	Membranous glomerulo-nephritis	Ischaemic heart disease Chronic bronchitis	Amphogel (aluminium hydroxide gel)	29	14	50
3	41	60	1	unknown	-	Amphogel	45	14	75
4	33	88	114	Goodpasture's syndrome or anti-glomerular basement membrane disease	Anephric	Alutab 6 3x daily	38	12	51

a. Normal range 36-55

b. Serum aspartate aminotransferase; normal range <50

c. Normal range 140-180

d. All patients also receiving: Ferrous Gluconate 300 mg/day
Calcitriol 0.25 µg/day
Vitamin C 50 mg 3x week
Vitamin B complex 3x week

enzyme is normally high in liver but low in blood. Damage to liver cells, caused by disease, liberates the enzyme causing a rise in the normal serum AST concentration. It was important to establish the absence of liver disease in the patients studied because of the major role the liver is believed to have in morphine metabolism. Serum albumin concentrations were in the normal range for all patients except one (2). Morphine in plasma, binds to this protein and the suggestion has been made[10] that reduced albumin concentrations during renal failure may cause unusually high unbound plasma morphine concentrations. All patients had lower than normal haemoglobin concentrations. Anaemia frequently accompanies renal failure and is likely to cause increased cardiac output[47]. This increase in cardiac output, and hence in the circulation of the blood, is a compensatory mechanism to maintain tissue oxygenation. The reduced haemoglobin concentrations observed suggested that the patients in this study had higher than normal blood flows.

The volunteers were three male physicians, aged 34-41 years. All were free of medical disease and were taking no regular medication.

2.5.2 Clinical Protocol

The study was approved by the Canterbury Hospital Board Ethical Committee, and all subjects gave written informed consent. All medical procedures were performed by medical staff at Christchurch Hospital. Papaveretum (0.25 mg/kg body weight) was injected into the deltoid muscle (a muscle in the upper arm). In the patients this was the fistula arm

(see note 3, Appendix B) Blood was obtained from a venous cannula (a tube inserted into a vein) in the contralateral arm immediately before injection and at intervals over the next 24 h. Samples were collected into plain tubes and the serum separated and stored at -20°C until assayed.

The clinical response following papaveretum injection for both patients and healthy volunteers ranged from vomiting and somnolence to mild elation and hyperactivity.

2.5.3 Analysis of Serum Samples

Serum samples from the patients and healthy volunteers were analysed for morphine by ammonia CI GCMS, using the procedures which have been described. The intensities of MH^+ ions from TMS_2-D_0-morphine (m/z 430) and TMS_2-D_3-morphine (m/z 433) were monitored for quantitation, and m/z 430 was monitored to rigorously establish morphine as the analyte. The samples were analysed in a random sequence and the time of sample collection was unknown at the time of analysis. No morphine was detected in the serum samples taken immediately before papaveretum administration. The results for the analysis of the serum samples, and the calibration data and statistical analysis of these data, are shown in Tables 6-16.

A calibration graph, at a total D_0 + D_3-morphine concentration of 60 ng mL^{-1} of plasma, was obtained and used on each occasion that unchanged morphine was measured. A similar, single, calibration graph, at a total D_0 + D_3-morphine concentration of 400 ng mL^{-1} of plasma was obtained for the determination of total (conjugated and unchanged) morphine in samples from two patients.

TABLE 6 CALIBRATION DATA FOR MORPHINE STUDY

Mass ratio	Calibration lines									
(D_0/D_3)	1	2	3	4	5	6	7	8	9	10
0.25	0.29	0.27	0.22	0.25	0.29	0.28	0.26	0.27	0.29	0.32
	0.26	0.22	0.29	0.30	0.28	0.28	0.29	0.25	0.27	0.19
								0.26	0.26	0.25
0.50	0.63	0.48	0.57	0.47	0.56	0.56	0.49	0.51	0.53	0.49
	0.53	0.57	0.45	0.56	0.55	0.61	0.55	0.47	0.48	0.44
								0.48	0.51	0.60
1.00	0.92	0.93	1.12	0.93	0.98	0.99	1.17	1.02	1.03	1.16
	1.04	1.12	1.20	0.98	1.07	1.06	0.92	0.96	0.94	0.89
								1.03	0.97	0.92
2.00	1.87	2.15	2.21	1.87	2.01	2.14	2.18	1.94	2.08	1.87
	1.97	2.08	2.14	2.19	1.91	2.00	2.13	2.01	1.93	2.21
								2.07	2.06	2.32

1-7 $D_0 + D_3$ = 60 $ngmL^{-1}$ extracted from plasma

8 $D_0 + D_3$ = 400 $ngmL^{-1}$ " " "

9 $D_0 + D_3$ = 200 $ngmL^{-1}$ " " "

10 $D_0 + D_3$ = 20 $ngmL^{-1}$ " " "

TABLE 7 REGRESSION ANALYSIS STATISTICS FOR CALIBRATION DATA

Calibration graph	r	Gradient	$100r^2$	Least squares Line	Theoretical value of r at 99.9%
1	0.9916	0.9484	98.32	y = 0.0523 + 0.9484x	0.9249
2	0.9939	1.0615	98.78	y = -0.0181 + 1.0615x	"
3	0.9929	1.1277	98.58	y = -0.0293 + 1.1277x	"
4	0.9918	0.9727	98.37	y = 0.0285 + 0.9727x	"
5	0.9982	0.9697	99.64	y = 0.0493 + 0.9697x	"
6	0.9966	1.0199	99.32	y = 0.0348 + 1.0199x	"
7	0.9934	1.0571	98.68	y = 0.0052 + 1.0571x	"
8	0.9983	0.9894	99.64	y = 0.0094 + 0.9894x	0.8233
9	0.9974	0.9781	99.48	y = 0.0250 + 0.9781x	"
10	0.9814	1.0489	96.31	y = -0.0141 + 1.0489x	"

TABLE 8 RESULTS FOR UNCHANGED MORPHINE IN SERUM SAMPLES FROM HEALTHY VOLUNTEER 1

Time after injection (min)	D_3-morphine conc. $ngmL^{-1}$ serum	Peak height ratio D_0/D_3	Calibration graph	Measured morphine conc. $ngmL^{-1}$ serum
15	30.0	1.11	2	31.9
30	"	1.83	"	52.2
45	"	1.57	"	45.0
60	"	1.23	"	35.3
75	"	1.16	"	33.3
90	"	1.11	"	31.9
105	"	1.09	"	31.3
120	"	1.02	"	29.3
150	"	0.98	"	28.2
180	"	0.78	"	22.6
210	"	0.63	"	18.3
240	"	0.48	"	14.1

TABLE 9 RESULTS FOR UNCHANGED MORPHINE IN SERUM SAMPLES FROM HEALTHY VOLUNTEER 2

Time after injection (min)	D_3-morphine conc. $ngmL^{-1}$ serum	Peak height ratio D_0/D_3	Calibration graph	Measured morphine conc. $ngmL^{-1}$ serum
15	30.0	0.93	1	27.8
30	"	1.14	"	34.4
45	"	0.99	"	29.7
60	"	0.87	"	25.9
75	"	0.92	"	27.4
90	"	0.93	"	27.8
105	"	0.86	"	25.5
120	"	0.81	"	24.0
150	"	0.68	"	19.9
180	"	0.56	"	16.1
210	"	0.46	"	12.9
240	"	0.43	"	11.9

TABLE 10 RESULTS FOR UNCHANGED MORPHINE IN SERUM SAMPLES FROM HEALTHY VOLUNTEER 3

Time after injection (min)	D_3-morphine conc. $ngmL^{-1}$ serum	Peak height ratio D_0/D_3	Calibration graph	Measured morphine conc. $ngmL^{-1}$ serum
2	30.0	0.46	7	12.9
5	"	1.42	"	40.2
10	"	1.09	"	30.8
15	"	1.15	"	32.5
20	"	1.41	"	39.9
25	"	1.06	"	29.9
30	"	1.19	"	33.7
35	"	1.35	"	38.2
40	"	1.11	"	31.4
45	"	1.15	"	32.5
55	"	1.03	"	29.1
70	"	0.94	"	26.5
80	"	0.94	"	26.5
100	"	0.73	"	20.1
130	"	0.67	"	18.9
180	10.0	1.39	10	13.4
240	"	0.85	"	8.2
360	"	0.65	"	6.3
480	"	0.25	"	2.5
1440	"	-	"	n.d.

n.d. none detected

TABLE 11 RESULTS FOR UNCHANGED MORPHINE IN SERUM SAMPLES FROM PATIENT 1

Time after injection (min)	D_3-morphine conc. $ngmL^{-1}$ serum	Peak height ratio D_0/D_3	Calibration graph	Measured morphine conc. $ngmL^{-1}$ serum
15	100.0	0.54	9	53.0
30	30.0	1.79	3	48.4
45	"	1.49	"	40.4
60	"	1.27	"	34.6
75	"	1.08	"	29.5
90	"	0.96	"	26.3
105	"	0.77	"	21.3
120	"	0.69	"	19.1
150	"	0.66	"	18.3
180	"	0.50	"	14.1
215	10.0	1.20	10	11.6
245	"	1.02	"	9.9
300	"	0.73	"	7.1
365	"	0.53	"	5.2
420	"	0.52	"	5.1
1430	"	–	"	n.d.

n.d. none detected

TABLE 12 RESULTS FOR UNCHANGED MORPHINE IN SERUM SAMPLES FROM PATIENT 2

Time after injection (min)	D_3-morphine conc. $ngmL^{-1}$ serum	Peak height ratio D_0/D_3	Calibration graph	Measured morphine conc. $ngmL^{-1}$ serum
19	30.0	1.66	5	49.8
30	"	1.04	"	30.6
45	"	1.15	"	34.1
60	"	1.02	"	30.0
75	"	0.68	"	19.5
90	"	0.64	"	18.3
105	"	0.62	"	17.7
125	"	0.61	"	17.3
150	"	0.55	"	15.5
180	10.0	1.02	10	9.9
210	"	0.93	"	9.0
240	"	0.80	"	7.8
300	"	0.62	"	6.0
360	"	0.55	"	5.4
420	"	0.43	"	4.2
480	"	-	"	n.d.
1425	"	-	"	n.d.

n.d. none detected

TABLE 13 RESULTS FOR UNCHANGED MORPHINE IN SERUM SAMPLES FROM PATIENT 3

Time after injection (min)	D_3-morphine conc. $ngmL^{-1}$ serum	Peak height ratio D_0/D_3	Calibration graph	Measured morphine conc. $ngmL^{-1}$ serum
5	30.0	1.31	6	37.5
10	"	1.38	"	39.6
15	"	1.23	"	35.2
30	"	1.61	"	46.3
45	"	1.46	"	41.9
60	"	1.05	"	29.9
75	"	0.77	"	21.6
90	"	0.81	"	22.8
105	"	0.74	"	20.7
120	"	0.74	"	20.7
150	"	0.57	"	15.7
210	10.0	0.96	10	9.3
240	"	0.84	"	8.1
300	"	0.56	"	5.5
360	"	0.43	"	4.2

TABLE 14 RESULTS FOR UNCHANGED MORPHINE IN SERUM SAMPLES FROM PATIENT 4

Time after injection (min)	D_3-morphine conc. $ngmL^{-1}$ serum	Peak height ratio D_0/D_3	Calibration graph	Measured morphine conc. $ngmL^{-1}$ serum
15	100.0	0.73	9	72.1
30	"	0.65	"	63.9
45	30.0	1.83	4	55.6
60	"	1.49	"	45.1
75	"	1.44	"	43.5
90	"	0.43	"	12.4
120	"	0.39	"	11.1
150	10.0	0.74	10	7.2
180	"	0.75	"	7.3
210	"	0.52	"	5.1
240	"	0.44	"	4.3
300	"	0.25	"	2.5

TABLE 15 RESULTS FOR CONJUGATED MORPHINE IN SERUM SAMPLES FROM PATIENT 1

Time after injection (min)	D_3-morphine conc. $ngmL^{-1}$ serum	Peak height ratio D_0/D_3	Calibration graph	Measured morphine conc. $ngmL^{-1}$ serum	Conjugated morphine conc. $ngmL^{-1}$ serum
15	200.0	0.37	8	72.9	19.9
30	"	0.73	"	145.7	97.3
45	"	0.87	"	174.0	133.6
60	"	1.08	"	216.4	181.8
75	"	1.11	"	222.5	193.0
90	"	1.22	"	244.7	218.4
105	"	1.21	"	242.7	221.4
120	"	1.16	"	232.6	213.5
150	"	1.37	"	275.0	256.7
215	"	1.52	"	305.34	293.8
245	"	1.50	"	301.3	291.4
300	"	1.47	"	295.2	288.1
365	"	1.45	"	291.2	286.0
420	"	1.44	"	289.1	284.0
1430	"	1.30	"	260.8	260.8

TABLE 16 RESULTS FOR CONJUGATED MORPHINE IN SERUM SAMPLES FROM PATIENT 2

Time after injection (min)	D_3-morphine conc. $ngmL^{-1}$ serum	Peak height ratio D_0/D_3	Calibration graph	Measured morphine conc. $ngmL^{-1}$ serum	Conjugated morphine conc. $ngmL^{-1}$ serum
19	100	1.17	9	117.1	67.3
30	"	1.41	"	141.6	111.0
60	"	1.86	"	187.6	157.6
90	200.0	1.02	8	204.3	186.0
105	"	1.17	"	234.6	216.9
125	"	1.18	"	236.6	219.3
180	"	1.16	"	232.6	222.7
240	"	1.31	"	262.9	255.1
360	"	1.31	"	262.9	257.5
420	"	1.24	"	248.8	244.6
480	"	1.31	"	262.9	262.9
1425	"	1.04	"	208.3	208.3

Samples found to have a $D_0 + D_3$ ratio outside the 0.25 - 2.0 range covered by these calibration graphs were analysed again, after spiking with a more appropriate amount of internal standard, and using a D_0- + D_3-morphine = 20 ng mL^{-1} or 200 ng mL^{-1} calibration graph. Samples found to have a D_0:D_3 ratio near the extremes of the calibration graphs were also re-analysed if sufficient serum was available.

A weighted linear least-squares procedure (Appendix C) was used to calculate the regression lines. The weighting factors used for the 60 ng mL^{-1} calibration graphs were those calculated for similar graphs obtained earlier (Section 2.4.7). These weights were based on five, rather than two, replicate determinations at each mass ratio, and were thus more accurate. The weighting factors used for the 20, 200 and 400 ng mL^{-1} graphs were obtained from the actual calibration data.

2.6 Pharmacokinetic Analysis

2.6.1 Selection of a Model For Unchanged Morphine

Various mathematical models can be used to simulate the processes of drug absorption, distribution and elimination in the body. These models provide equations to which experimental data can be fitted, allowing calculation of pharmacokinetic parameters.

The simplest pharmacokinetic model is the "one-compartment-open model" in which the body is considered to be a single compartment. The model assumes that drug in the plasma reaches instantaneous equilibrium with drug in the tissues and that any changes occurring in the plasma

concentration reflect proportional changes in tissue drug levels. Drug elimination from the single compartment follows first order kinetics, giving a simple monophasic decline in drug concentration.

Some drugs require a more sophisticated model to explain their plasma concentration-time curve. The "two-compartment-open model" assumes that a drug distributes into two compartments. The first or central compartment represents the blood, extracellular water and highly perfused tissues which the drug enters rapidly, and the second or peripheral compartment represents tissues that the drug enters more slowly. The two-compartment model predicts a biphasic decline in plasma concentrations during drug elimination. The initial and rapid α phase or distribution phase corresponds to drug in the first compartment entering the second compartment. After distribution, the decline in drug concentration enters the slower β phase or elimination phase, representing elimination of drug from the central compartment.

With both these models, and more complicated multicompartment models, drug can enter the system instantaneously (corresponding to a rapid intravenous injection), by a zero-order process (corresponding to an intravenous infusion) or by a first-order process (corresponding to absorption from the gastrointestinal tract or any other extravascular site).

In this work serum unchanged morphine concentration data for each subject was analysed by computer using the non-linear least-squares regression analysis program

NONLIN[48]. The data points were fitted, without weighting, to the following two functions.

$$Cp = A(e^{-\beta t} - e^{-kat}) \qquad (1)$$

$$Cp = A(e^{-\beta t} - e^{-kat}) + Be^{-\gamma t} \qquad (2)$$

The quantity Cp represents the serum morphine concentration at time t, and A, B, ka, β and γ are coefficients which were varied iteratively by the program to give curves of best fit, determined by comparison of the sum of squares of the residual errors. These two functions represent one and two compartment open pharmacokinetic models respectively. The choice between equations (1) and (2) as functions of best fit to the experimental data for each subject was determined by a standard F-ratio test and the criterion that the model selected be the one with the fewest number of compartments which are necessary to adequately describe the experimental data. For all subjects there was no advantage gained by using equation (2) over the simple equation (1).

2.6.2 Pharmacokinetic Parameters For Unchanged Morphine

All pharmacokinetic parameters for unchanged morphine were calculated using conventional methods[49]. The coefficient ka in equation (1) represents the first-order rate constant for absorption of morphine into the systemic circulation from the site of injection. These coefficients were used in equation (3) to calculate values for the absorption half life ($t_{\frac{1}{2}}$abs)

$$t_{\frac{1}{2}}\ abs = \frac{\ell n 2}{ka} \qquad (3)$$

The coefficient β in equation (1) represents the first-order rate constant for morphine elimination. This elimination may be due to drug metabolism or excretion. The coefficients were used in equation (4) to calculate values for the elimination half-life ($t_{½}$ elim), which is the time for the amount of morphine in the systemic circulation to fall to half of that initially present.

$$t_{½}\ \text{elim} = \frac{\ell n 2}{\beta} \qquad (4)$$

Two other pharmacokinetic parameters were calculated for each subject, the total body clearance (CL) and the apparent volume of distribution (V). Clearance, like $t_{½}$ elim, characterises drug elimination, and is the volume of blood or plasma cleared of drug per unit time. For most drugs the clearance is constant over the plasma or blood concentration normally found: that is, elimination is not saturable, and the rate of elimination is directly proportional to concentration. Clearance values were calculated using equation (5).

$$CL = \frac{F.D}{AUC} \qquad (5)$$

The quantity F is the fraction of the administered morphine dose absorbed which reached the general circulation. The systemic availability of I.M. morphine has been shown to be 100%[50], and F was assumed to be 1. D is the dose of morphine administered. The usual form of morphine used in clinical practice is morphine sulphate, rather than anhydrous morphine as contained in papaveretum, so D was expressed as morphine sulphate equivalents. AUC is the area under the plasma morphine concentration versus time curve determined by the least-squares regression analysis. Values of AUC for each subject were calculated using equation (6),

$$AUC = A(\frac{1}{\beta} - \frac{1}{ka}) \qquad (6)$$

where A, β and ka are as defined earlier.

The apparent volume of distribution is that volume of fluid into which a drug appears to distribute with a concentration equal to that in plasma. The volume, which does not necessarily correspond to a real anatomic volume (e.g. the blood volume), gives an indication of the extent to which the drug passes from the plasma into the tissues. Values of V for each subject were calculated using equation (7),

$$V = \frac{CL}{\beta} \qquad (7)$$

where Cl and β are as previously defined.

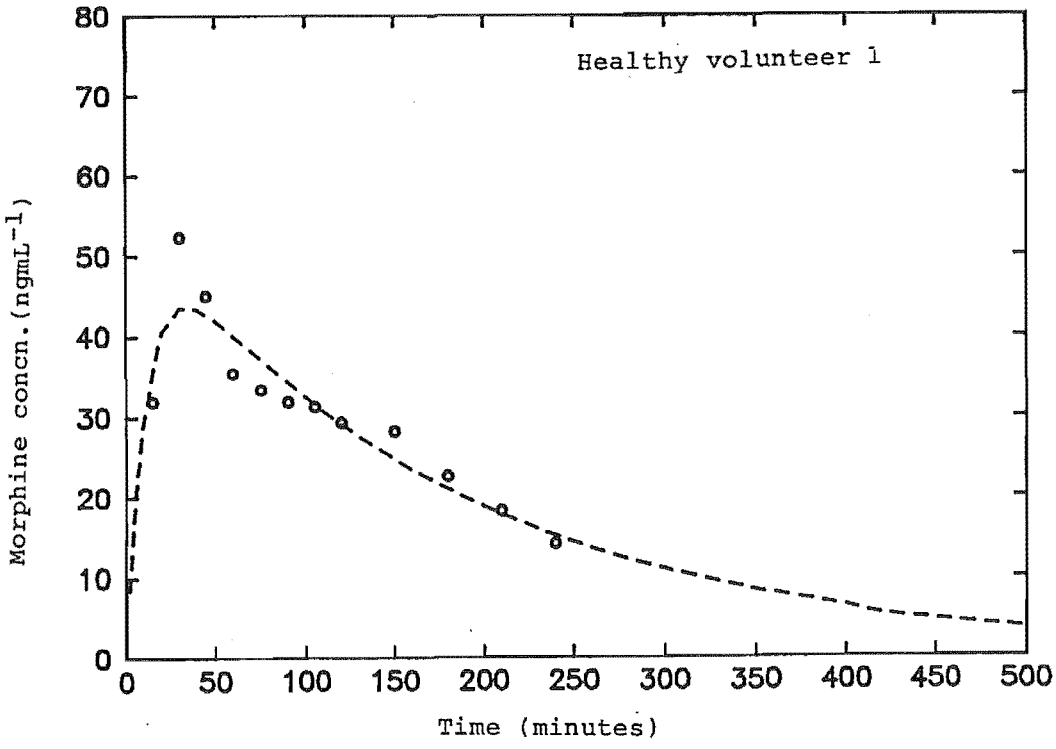
Table 17 gives the dose of morphine administered (expressed as morphine sulphate equivalents), the coefficients to equation (1) calculated by NONLIN, and the derived pharmacokinetic parameters for each subject. Figure 10 shows unchanged morphine concentration versus time curves, fitted by computer, for the healthy volunteers and the renal failure patients.

2.6.3 Statistical Analysis For Unchanged Morphine

Pharmacokinetic parameters for the healthy volunteer and renal failure groups were compared using unpaired Student's t-test (2-tailed). The mean half life of morphine absorption ($t_{\frac{1}{2}}$ abs) and the mean half life of morphine elimination ($t_{\frac{1}{2}}$ elim) were significantly shorter in the patients compared with the healthy volunteers ($p < 0.05$). The difference between the two groups in mean clearance (CL) and in mean volume of distribution (V) corrected for body weight did not reach statistical significance.

Table 17 Pharmacokinetic parameters of papaveretum disposition after intramuscular administration.

Healthy Volunteers	Dose (mg)	A (μg/L)	ka	β	$t_{½}$ abs (h)	$t_{½}$ elim (h)	AUC ($\mu gL^{-1}h$)	CL ($Lh^{-1}kg^{-1}$)	V (Lkg^{-1})
1	13.3	55.9	5.28	0.3255	0.13	2.13	161.18	1.03	3.17
2	15.3	39.24	6.29	0.2865	0.11	2.42	130.74	1.30	4.54
3	13.3	40.08	19.84	0.3515	0.03	1.97	112.02	1.52	4.33
Mean					0.09	2.17	134.62	1.28	4.01
± s.e.m.					0.03	0.13	14.32	0.14	0.43
Renal Failure Patients									
1	13.1	58.52	31.22	0.495	0.02	1.40	116.37	1.22	2.92
2	11.3	48.58	22.77	0.526	0.03	1.32	90.16	2.17	4.14
3	10.0	48.43	17.06	0.455	0.04	1.52	103.71	1.59	3.51
4	14.7	128.77	6.14	1.1015	0.11	0.63	95.92	1.73	1.57
Mean					0.05	1.21	101.54	1.67	3.05
± s.e.m.					0.02	0.20	5.67	0.19	0.55



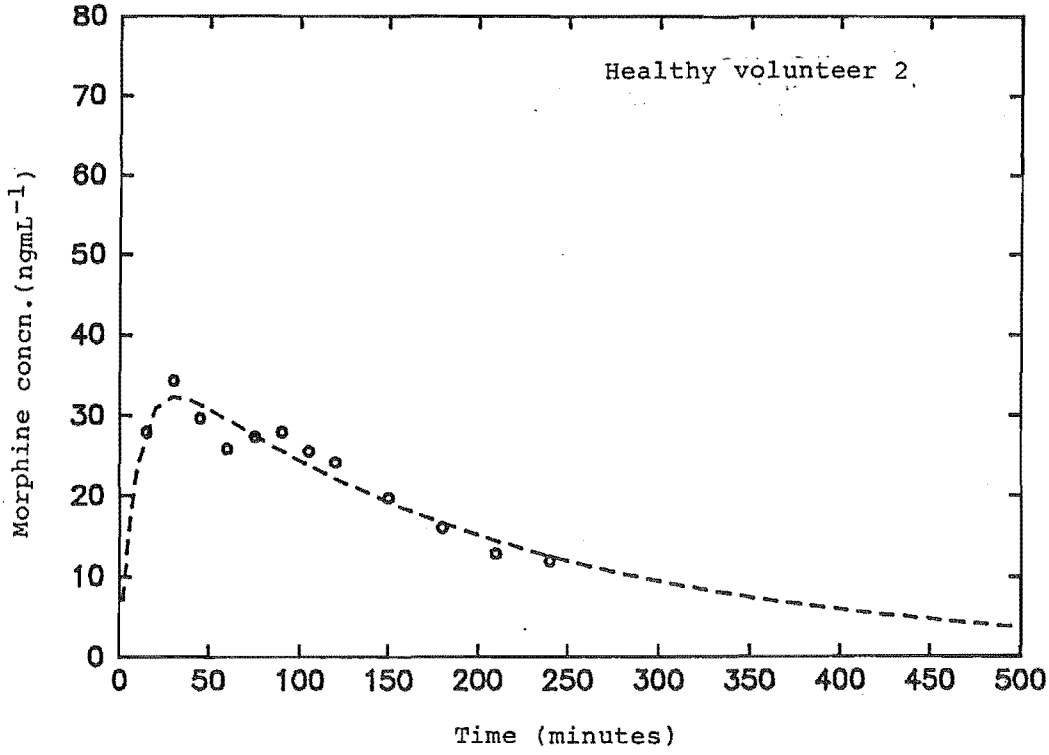
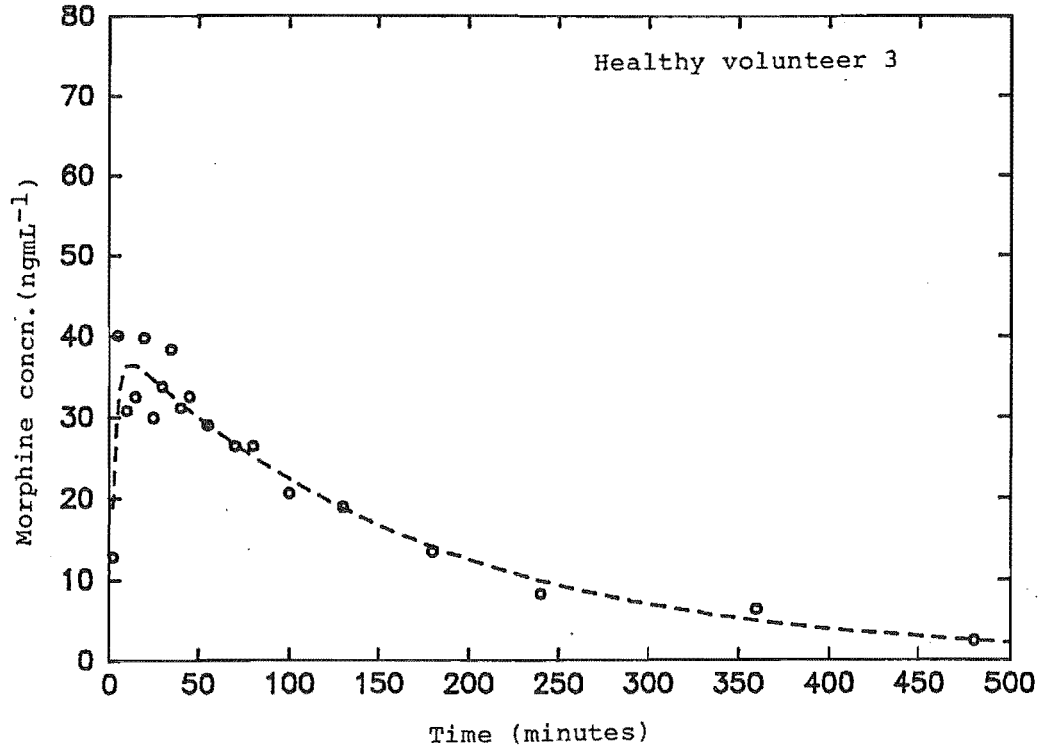


Figure 10a. Measured serum morphine concentrations (o) and the computer-fitted curve (---) for healthy volunteers 1 and 2.



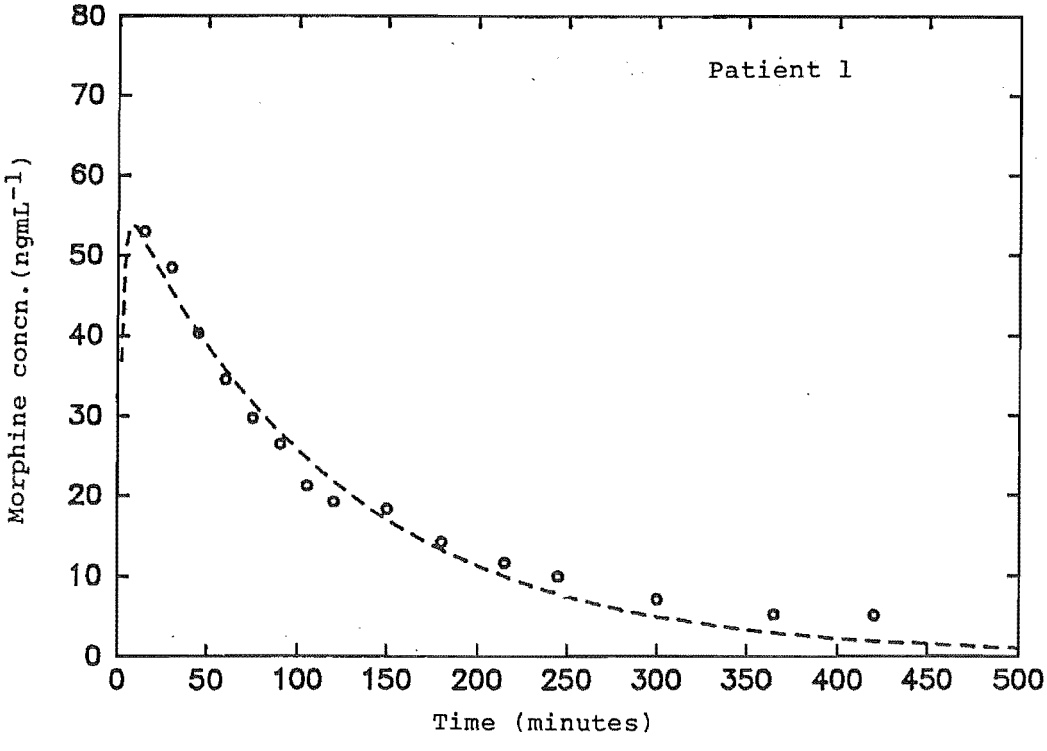
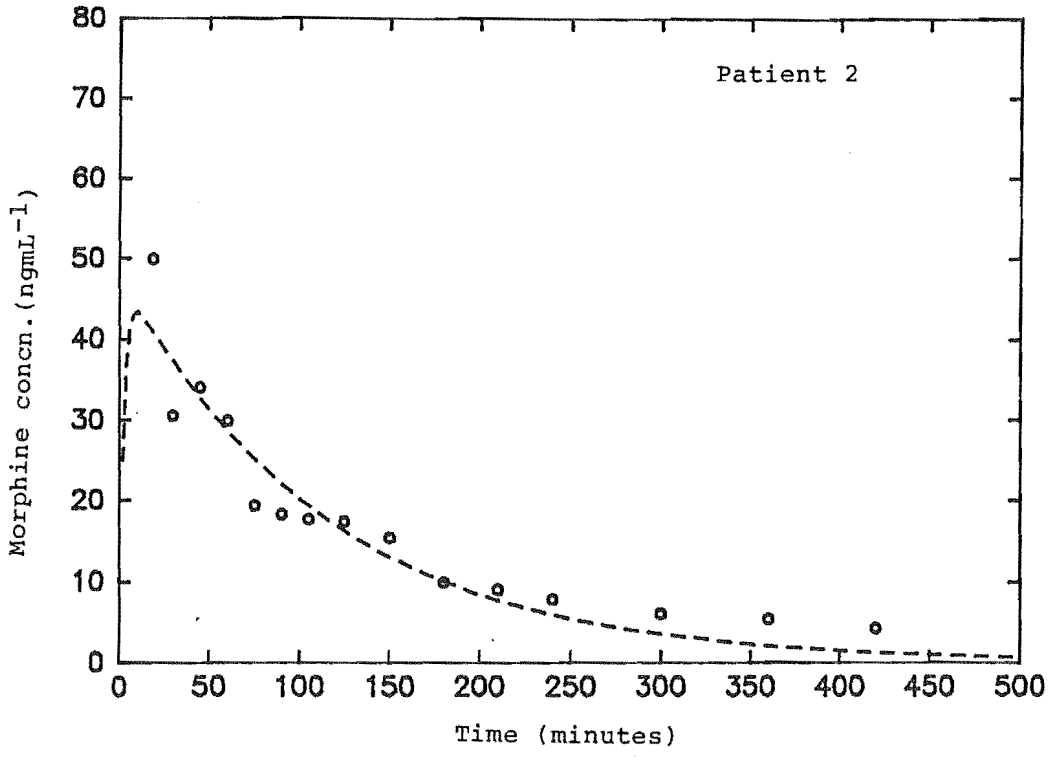


Figure 10b. Measured serum morphine concentrations (o) and the computer-fitted curve (---) for healthy volunteer 3 and patient 1.



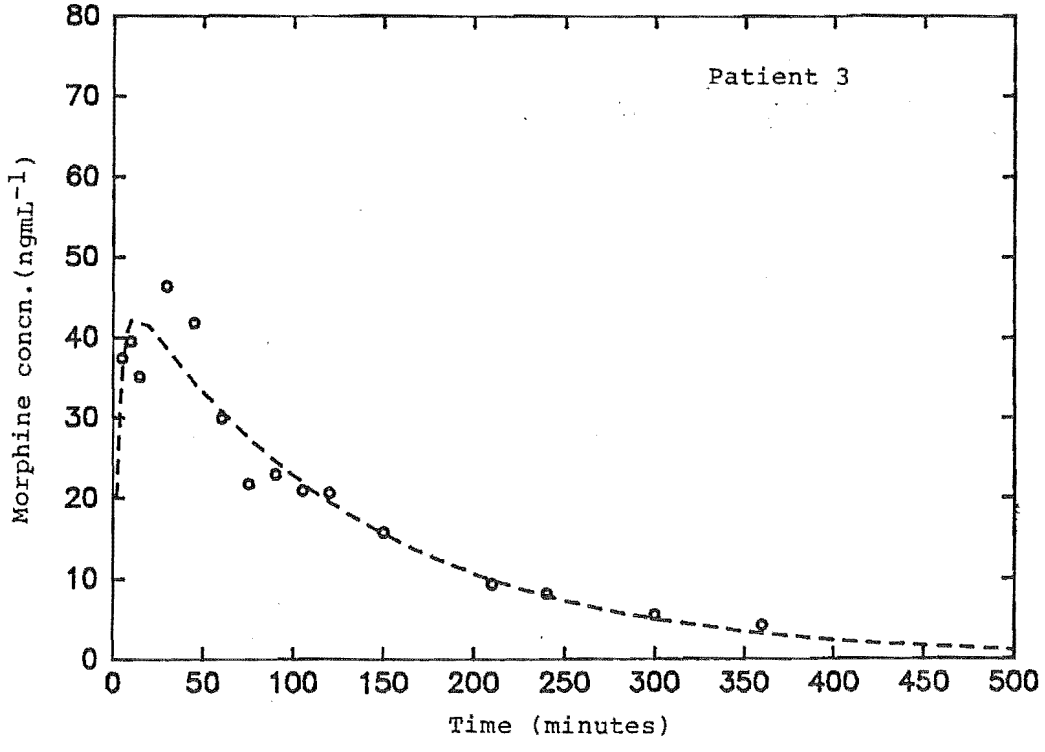


Figure 10c. Measured serum morphine concentrations (o) and the computer-fitted curve (---) for patients 2 and 3.

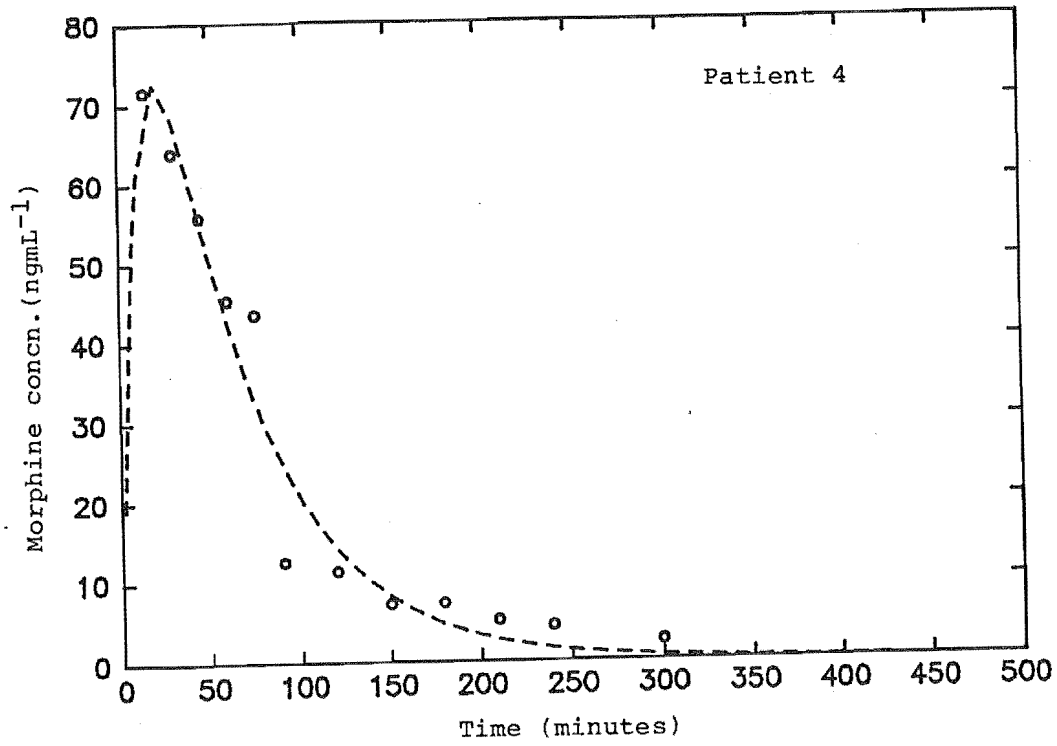


Figure 10d. Measured serum morphine concentrations (o) and the computer-fitted curve (---) for patient 4.

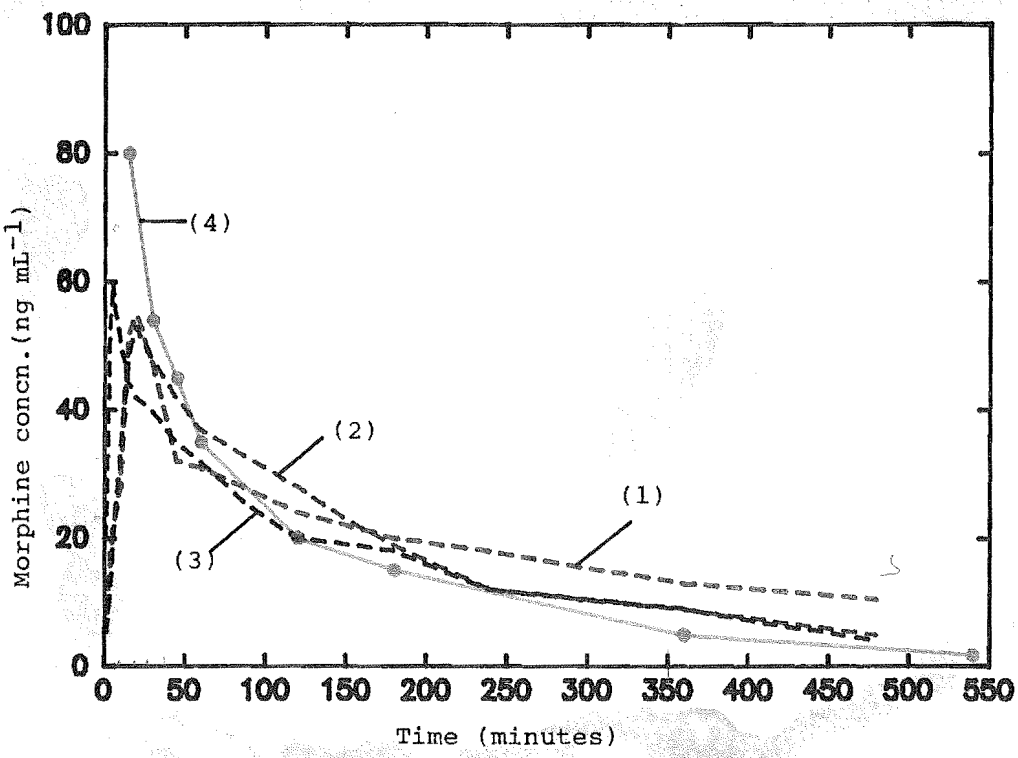


Figure 11. Plasma morphine concentration versus time curves reproduced from the literature.

2.6.4 Pharmacokinetic Interpretation

(a) Unchanged Morphine

The literature contains several plasma unchanged morphine concentration versus time curves for healthy subjects who have been administered I.M. morphine[50,51]. These are shown in Figure 11 as plots reproduced from the published curves using a grid to accurately read off the data points. Curves 1-3 are computer fitted curves that represent pharmacokinetic functions determined by non-linear least squares regression analysis which describe the plasma morphine concentrations at various times for three subjects who each received 10 mg of morphine sulphate. Morphine concentrations were measured by RIA. Curve 4 is a line which was drawn through mean measured plasma morphine concentrations for six subjects who each received 5.75 mg of N-methyl-^{14}C morphine sulphate per square metre of body surface area. Morphine concentrations were determined by measuring the radioactivity of extracted plasma samples. Comparision of the pharmacokinetic curves calculated for unchanged morphine in the healthy volunteers in the present study with the literature curves shows that unchanged morphine concentrations for the healthy volunteers in this study were in the same range as those in the earlier reports. In this study morphine absorption into the systemic circulation from the site of injection was altered in the patients with renal failure. The shorter absorption half life shows that absorption was more rapid in the patients than in the healthy subjects. The most likely reason for the observed increase in the rate of morphine absorption in the patients is an increased blood flow at the

site of injection which would transfer drug into the systemic circulation more rapidly than normal. Cardiac output is likely to be greater in patients with renal failure[47] and there was reason to believe that the patients in this study had higher than normal blood flows (section 2.5.1). Papaveretum was injected I.M. into the fistula arm of the patients, and blood flow is also likely to be greater in an arm with an arteriovenous fistula.

The rate of morphine elimination was also altered in the patients with renal failure. The shorter elimination half life for the patients shows that this group eliminated morphine more rapidly than the healthy volunteers. Drug elimination half life is dependent on both the ability to eliminate drug (clearance) and the volume of distribution. This can be seen from rearrangement of equations 4 and 7:

$$t_{\frac{1}{2}}elim = \frac{\ell n 2V}{CL} \qquad (8)$$

The mean volume of distribution was smaller and the mean clearance higher for the patients than for the healthy volunteers in this study. Unfortunately it was not possible to establish whether a change in the volume of distribution or a change in the clearance was responsible for the decrease in the elimination half life because the difference in the mean volume of distribution and in the mean clearance for the two groups did not reach statistical significance. Renal failure is known to cause a significant change in the volume of distribution for a number of drugs[52]. Changes in volumes of distribution are most often caused by unusually low binding of drugs to plasma proteins. The binding of many acidic drugs is decreased and the binding of many basic drugs is increased in renal failure. These changes in

binding are usually due to a combination of a decrease in serum albumin and a decrease in the binding capacity of the albumin due to a change in molecular configuration or an accumulation of an endogenous inhibitor. During renal failure there is excessive retention of protein metabolism by-products in the blood, a condition termed uremia. These endogenous substances, normally excreted in the urine, but retained in renal failure, can compete with drug at drug receptor sites on proteins and in tissues. A decrease in the protein binding of drugs which are normally highly protein bound makes more free (unbound) drug available for binding to the tissues and for possible clearance by the liver (but see below) thus decreasing the total (bound plus unbound) concentration of drug in the circulation and increasing the volume of distribution. Drugs which are not highly protein bound usually have little or no change in their volume of distribution. The protein binding of morphine in uremic patients has been shown to be impaired by lowered concentrations of plasma proteins and albumin[10]. Because all patients except one in this study had serum albumin concentrations in the normal range (Table 5), and the extent of morphine's binding to plasma proteins is normally small (30%)[10], changes in the protein binding of morphine in the patients studied are unlikely to have contributed to a change in the mean volume of distribution for this group. Impairment of drug binding at tissue sites would be expected to cause a decrease in the volume of distribution for drugs which are bound to a significant extent to tissues but which are not bound to a significant extent to plasma proteins. With one exception[53], there is

little information on this point in the literature. Impairment of morphine binding at tissue sites due to retained endogenous substances, may have had some effect on the volume of distribution for the patients in this study, although this is unlikely because all patients were on maintenance haemodialysis.

Clearance is a measure of the efficiency with which a drug is removed from the body. The sum of individual organ clearances equals the total drug clearance from the systemic circulation. Severe renal failure could be expected to reduce, or even prevent, drug clearance (by excretion and possibly metabolism) by the kidney, leaving the liver as (possibly) the main eliminating organ. Drugs which are efficiently eliminated from the blood by the liver (drugs with a high hepatic extraction ratio) have a clearance which depends on blood flow to the liver, and which is independent of any drug-protein binding. The clearance of drugs which are not efficiently extracted by the liver depends on the extent of drug-protein binding but is insensitive to changes in liver blood flow. That is, clearance depends on the liver's intrinsic clearance and not on the rate of drug presentation. Morphine has been reported to have a high hepatic extraction ratio (0.6-0.8) in healthy subjects[50], so a change in blood flow but not a change in the extent of its protein binding is likely to alter its clearance by the liver via conjugation with glucuronic acid. The patients in this study probably had higher than normal blood flows, so that morphine clearance by the liver may have been increased.

(b) Conjugated Morphine

Conjugated morphine concentrations were measured in the serum samples from patients 1 and 2. The conjugated morphine concentration versus time curves for these two patients are presented in Figure 12. The Figure also shows a mean plasma conjugated morphine concentration versus time curve reproduced from the literature[51] for six healthy subjects who each received 5.75 mg of morphine sulphate per square metre of body surface area. The Figure shows that morphine glucuronides accumulated in the patients in the present study, and that high concentrations of metabolite(s) were present even after 24 hours following papaveretum administration. Morphine glucuronides in blood are excreted primarily by the kidney into the urine in heathly subjects. Some non-renal elimination also occurs via the faeces and via enterohepatic recirculation (see note 4, Appendix B). Renal failure would thus be expected to cause accumulation of the conjugated metabolites, assuming the rate of conjugate formation is unaltered from normal.

2.6.5 This Work and the Renal Hypothesis

Morphine concentrations in the serum samples from the subjects who took part in this study were measured by a highly sensitive and specific analytical method, GCMS with QSIM. The results show that renal failure did not impair the elimination of morphine in the patients. The elimination half lives for morphine were not prolonged, and were actually slightly shorter than for the healthy subjects. It is worth noting that the anephric patient had

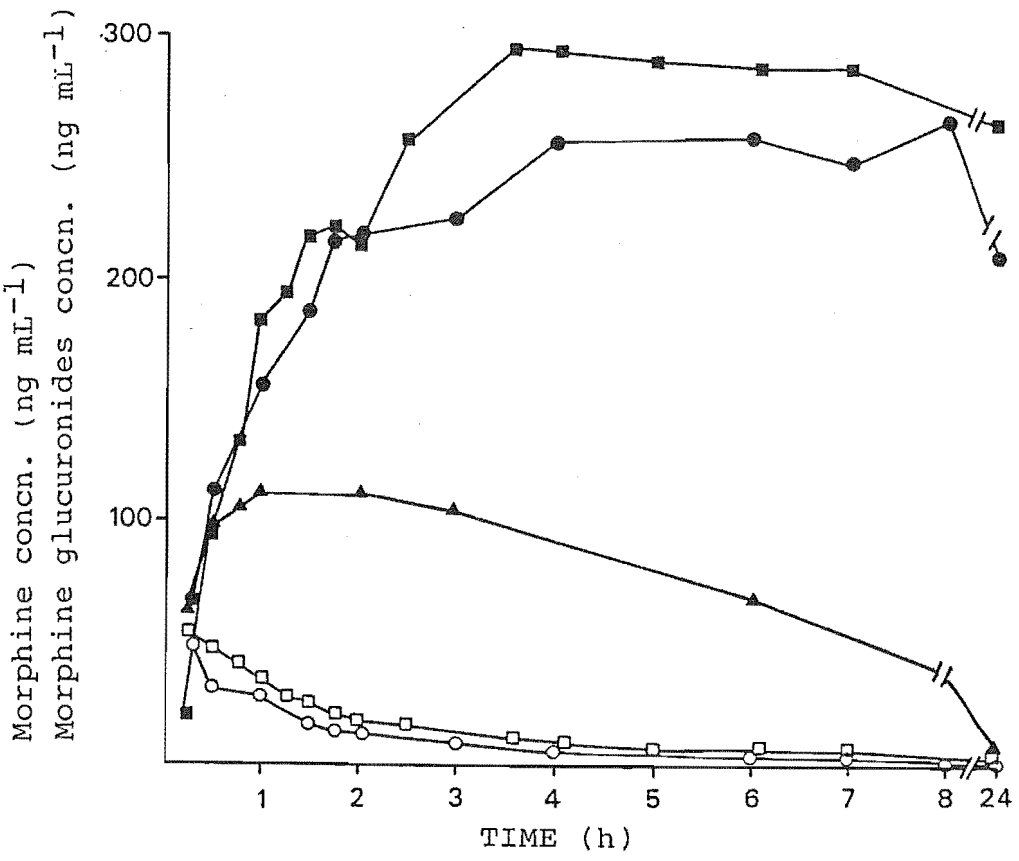


Figure 12. Plot of morphine (O and □) and morphine glucuronides (● and ■) concentrations for patients 1 and 2, and of mean morphine glucuronides concentrations for six healthy subjects reproduced from the literature.

the shortest elimination half life. Morphine glucuronides, measured in two patients, were eliminated slowly.

The renal hypothesis questions the traditional belief that the liver is the main site of morphine metabolism, and suggests that the kidney may have the major role. Morphine elimination and production of morphine metabolites should both be impaired in renal failure patients if the renal hypothesis is valid. The results from this work show that morphine elimination is not impaired by renal failure and strongly suggest that the renal hypothesis is not correct.

The studies that have reported impaired morphine elimination in patients with renal insufficiency, and on which the renal hypothesis is largely based, have all used an RIA method to measure morphine concentrations. Results based on immunoassay determinations of morphine should be viewed with caution. The antisera used in immunoassays for morphine are unlikely to be completely specific for this drug and may cross-react to some extent with morphine metabolites. The antisera used by McQuay and Moore had a cross-reactivity with morphine-3-glucuronide of less than 1%. The cross-reactivity with morphine-6-glucuronide was not investigated. Figure 12 suggests that shortly after morphine administration the concentration of conjugated morphine metabolites in serum from renal failure patients greatly exceeds the concentration of the parent drug, so that even a low level of cross-reactivity may lead to an overestimation of true morphine concentrations. Cross-reactivity by a morphine antiserum could be reduced, or even eliminated, by separating morphine from potentially interfering metabolites by extraction before the immunoassay

determination is performed. The disadvantage of this procedure is that an unknown recovery of drug will be obtained.

Other evidence has been used to support the renal hypothesis. The oral bioavailability of a controlled-release morphine tablet has been measured and found to be 100%[54]. Oral bioavailability indicates the relative amount of an oral drug dose reaching the systemic circulation as unchanged drug, and depends on the dosage form of the drug (solubility, particle size etc.), and the drug's metabolism. After oral administration morphine (and other drugs) may be metabolised in the gut, the gut wall, the portal blood or the liver prior to entry into the systemic circulation. An oral bioavailability for morphine of less than 100% would be predicted if significant pre-systemic metabolism of morphine by the liver occurs. The study reporting an oral bioavailability of 100% for morphine used RIA to measure morphine concentrations, and cross-reactivity by the antiserum with conjugated morphine metabolites may have caused the estimate of bioavailability to be erroneously high. Other measurements of morphine oral bioavailability have produced figures in the range 15-64%[55] (morphine concentrations measured by gas chromatography). A significant difference exists between the relative potency of orally and parenterally administered morphine (30 mg orally is required for an analgesic action equivalent to that of 10 mg by injection). As has been noted in the literature, there is no simple explanation for this difference if the kidney is the major site of morphine metabolism.

The results of an investigation of morphine elimination in patients with liver disease have been interpreted as evidence that an important extrahepatic site for morphine metabolism may exist[22] (although this site need not necessarily be the kidney). Patients with moderate to severe cirrhosis who were administered I.V. morphine were found to eliminate morphine and conjugated morphine normally. Plasma morphine concentrations, both before and after enzyme hydrolysis, were measured by gas chromatography, so lack of specificity for morphine, such as can occur with RIA, is less likely to have been a problem. A number of other drugs which are metabolised in the liver by conjugation with glucuronic acid, for example lorazepam[56] and oxazepam[57] (drugs with low hepatic clearances), have also been found to have normal clearances in patients with liver disease. A possible explanation for these observations may be that the enzymes involved in glucuronidation are located deep within liver microsomes, making them less susceptible to liver damage. Another possible explanation may be that glucuronidating enzyme activity is not diminished by liver disease. Experiments with rats have shown that damaged liver tissue can have unaltered or even increased glucuronyl transferase activity[58,59]. A difficulty with both of these explanations is that a frequent consequence of chronic liver disease is the development of intra- and extrahepatic vascular shunts that may carry drug in the portal circulation directly into the systemic circulation, reducing drug presentation to the liver's conjugating enzymes. There was evidence to suggest that such shunts existed in the patients in the morphine

metabolism-cirrhosis study, so the finding of normal morphine elimination makes it difficult not to postulate an extrahepatic site for morphine conjugation.

The results from the present study do not explain the anecdotal observations made by many nephrologists of delayed recovery from the effects of morphine in patients with renal failure. Because morphine does not accumulate in these patients it is unlikely to be responsible for the prolonged duration of drug action. Morphine glucuronides do accumulate, but morphine-3-glucuronide is believed to be pharmacologically inactive in humans[15]. Morphine-6-glucuronide may have considerable analgesic activity[14,60,61], and this metabolite may be present in the plasma of renal failure patients at concentrations exceeding those of morphine itself. In this study no attempt was made to distinguish between morphine-3-glucuronide and morphine-6-glucuronide when determining morphine metabolites conjugated with glucuronic acid. The glucuronide concentrations determined for two patients will almost certainly represent the total concentration of these two compounds. The presence of morphine-3-glucuronide did not affect determinations of morphine, and it is extremely unlikely that the presence of morphine-6-glucuronide would have any affect; morphine-6-glucuronide will be highly water soluble, and the results themselves suggest freedom from interference.

2.7 Conclusion

This study has shown that morphine elimination is not impaired in patients with renal failure after the I.M. administration of papaveretum, and that morphine glucuronides are eliminated slowly in these patients. The results suggest that renal failure patients may eliminate morphine more rapidly than healthy subjects. This study has been unable to confirm the observation that patients with renal failure have prolonged half lives for morphine elimination. Immunoassay determinations of morphine are unreliable and probably overestimate morphine concentrations because the antisera cross-react with conjugated morphine metabolites. The renal hypothesis of morphine metabolism is probably not valid, and some other explanation is required to rationalise the prolonged duration of morphine's action in patients with renal failure. The major site of morphine metabolism is also still uncertain. More work is required to establish this site to enable rational dosing of patients with this valuable analgesic drug.

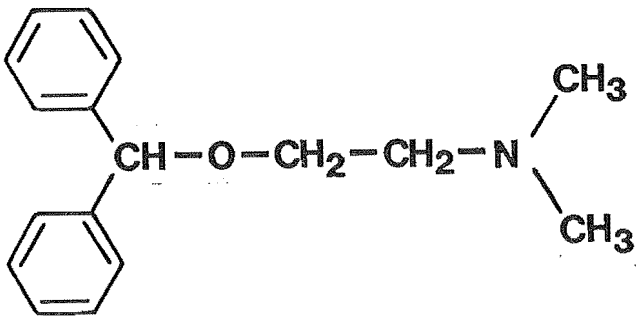
CHAPTER 3

INTRODUCTION

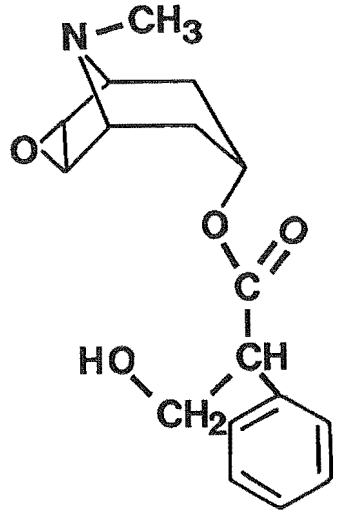
3.1 Benztropine

Benztropine mesylate ((3-diphenylmethoxy)-8-methyl-8-azabicyclo[3.2.1]-octane methanesulfonate), is a synthetic tertiary amine antiparkinsonian drug. It resembles both atropine and diphenhydramine in chemical structure and exhibits anticholinergic, antihistaminic and local anaesthetic properties. Benztropine was first suggested as a therapeutic agent after the discovery that some antihistaminic drugs (e.g. promethazine and diphenhydramine) in addition to the belladonna alkaloids atropine and scopolamine, produce a beneficial effect in parkinsonism. New drugs, including benztropine, were synthesised in an attempt to combine both atropine-like and antihistamine activities in the same molecule. These drugs generally contain structural features of atropine, and a diphenylmethane moiety or similar structural grouping which resembles that of the diphenhydramine group of antihistamines[62,63] (Figure 13).

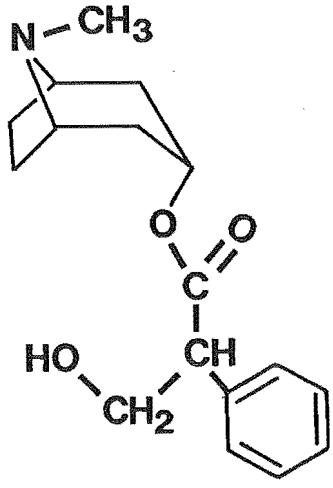
Benztropine is used to treat the symptoms of all forms of parkinsonism, including Parkinson's disease (paralysis agitans) and the parkinsonian-like effects induced by many antipsychotic drugs, especially phenothiazine derivatives. These drugs are used to treat severe psychotic illness and as a side-effect induce a parkinsonian-like syndrome which may be indistinguishable from paralysis agitans. Benztropine may be given alone or in conjunction with other



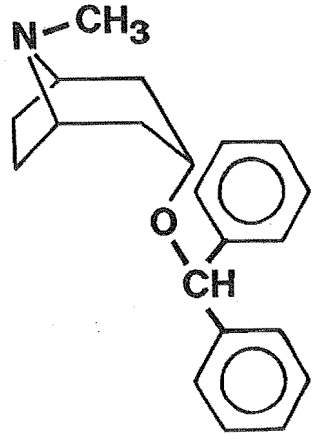
Diphenhydramine



Scopolamine



Atropine



Benztropine

FIGURE 13. BENZTROPINE AND RELATED DRUGS

drugs, and may produce a number of adverse side-effects, for example dryness of mouth, blurred vision and urinary retention.

Benztropine is dispensed under the proprietry names Benztropine, Benztropine mesylate, Congentin and Congenitol. It is available, by prescription, in tablets of 0.5, 1 and 2 mg, and in a 1 mg mL^{-1} solution for intramuscular or intravenous injection. The normal therapeutic daily dose is initially 0.5 to 1 mg and this is gradually increased up to 2-6 mg[64].

There have been several reports describing the clinical features of therapeutic and excessive benztropine dosage[65,66] but very little information is available about concentrations in body fluids and organs. Plasma and urine concentrations of 79.5-125.6 ng mL^{-1} and 5.3-123 ng mL^{-1} respectively have been measured in four psychiatric patients receiving antipsychotic medication and 2 mg benztropine twice daily[67]. There have been no reported pharmacokinetic studies for benztropine.

3.2 Analytical Methods for Benztropine

A spectrophotometric method for determining diphenhydramine and other compounds having a diphenylmethyl ether group, and thus applicable to benztropine, was described in 1975[68]. Acid hydrolysis of the ether linkage followed by oxidation gave benzophenone derivatives having a higher ultra-violet (UV) absorption coefficient than the original compounds, thus lowering the detection limit. The method was sufficiently sensitive to determine drug concentrations in urine after therapeutic dosages.

Gas chromatography using electron capture detection (e.c.d.) has been investigated as a method to assay diphenyl substituted compounds[69]. The electron capture sensitivity was enhanced by oxidising the compounds to benzophenone derivatives prior to chromatography. The determination of benztropine was not specifically considered but the method would appear sufficiently sensitive to detect the drug at nanogram concentrations. A problem with methods using oxidation is obtaining a high and reproducible yield of the benzophenone product, and a number of oxidising agents have been investigated[70,71].

Benztropine and two other anticholinergic agents have been included in an investigation of HPLC conditions suitable for the quantitative determination of common neuroleptic and antihistamine drugs in plasma[72-74]. The anticholinergics are likely to be coprescribed with these drugs and may be present in the plasma samples being analysed. The conditions of the assay were optimised for measuring the neuroleptics and antihistamines by u.v., and later, electrochemical detection. Quantitation of the anticholinergics was not attempted but benztropine was reported to give only a small signal.

The first and only assay specifically designed for determining benztropine was recently reported by Jindal et al[67]. Using gas chromatography-mass spectrometry (GCMS), with the mass spectrometer operating under electron-impact conditions, benztropine concentrations in plasma and urine were found by measuring the ratio of specific fragment ion intensities from benztropine and a S.I.A. internal standard, trideuteriomethylnorbenztropine ([$^2H_3$]-benztropine). The

assay could measure 5 ng mL^{-1} with about 6% precision and was used to measure drug concentrations in plasma and urine samples from patients on benztropine therapy.

3.3 This Work

A GCMS procedure with electron impact ionisation has been reported for determining benztropine in plasma and urine. This section of the thesis describes a method for determining benztropine by GCMS with ammonia chemical ionisation mass spectrometry. $[^2H_3]$-benztropine was synthesised by a new method and used as an internal standard. The assay was developed for measuring benztropine concentrations in post-mortem blood and for detecting benztropine in post-mortem liver samples submitted for analysis to the Christchurch Laboratory of the Chemistry Division of the Department of Scientific and Industrial Research (DSIR). This laboratory is responsible for providing an analytical service for the Coroners in its area. The development of the assay was prompted by reports from the laboratory of difficulty in detecting benztropine using its normal drug screening procedures.

RESULTS AND DISCUSSION

3.4 Discussion of Preparative Procedures

(a) Benztropine

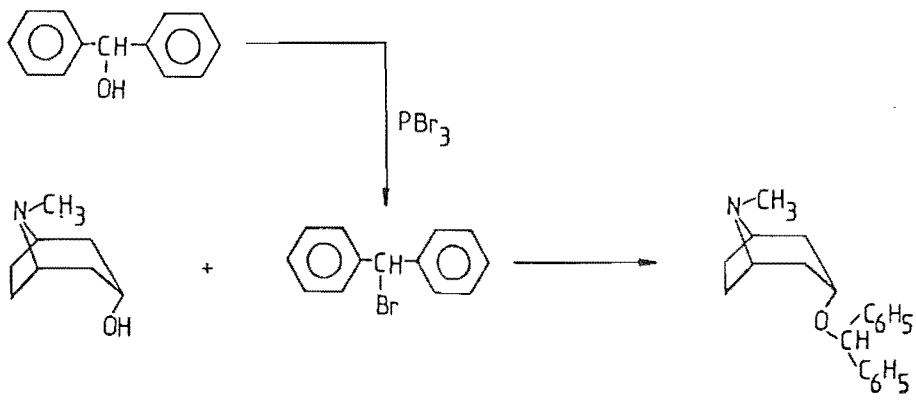
Benztropine was prepared by the reaction of bromodiphenylmethane with tropine[75], and the crude product purified by formation of its hydrobromide followed by repeated recrystallisation from water. Bromodiphenylmethane was formed by reaction of benzhydrol with phosphorus tribromide[76] (Figure 14, scheme 1).

(b) N-Trideuteriomethylnorbenztropine

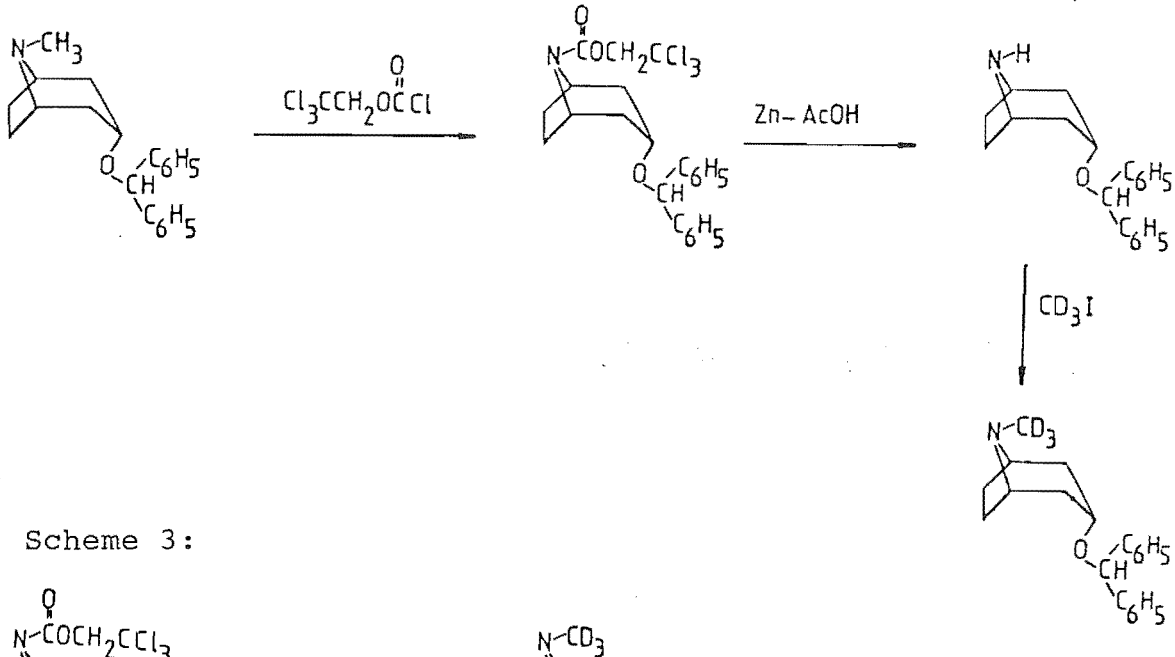
Jindal *et al*[67] have previously prepared N-trideuteriomethylnorbenztropine by the reaction of norbenztropine with D_3-methyliodide. Norbenztropine was formed by reaction of benztropine with 2,2,2-trichloroethylchloroformate to form N-(2,2,2-trichloroethoxycarbonyl)-norbenztropine which was then treated with zinc dust and acetic acid (Figure 14, scheme 2).

Attempts to duplicate this synthesis were unsuccessful. Formation of the carbamate proceeded smoothly, but all attempts to reintroduce the methyl group failed. An alternative route was therefore considered. Abdel-Monem and Portoghese[77] have previously shown that treatment of morphine with phenylchloroformate gave the carbamate which could then be reduced with lithium aluminium deuteride (LAD) to trideuteriomorphine. Based on this, N-(2,2,2-trichloroethoxycarbonyl)-norbenztropine was treated with LAD under reflux in dry tetrahydrofuran and the reaction followed by

Scheme 1:



Scheme 2:



Scheme 3:

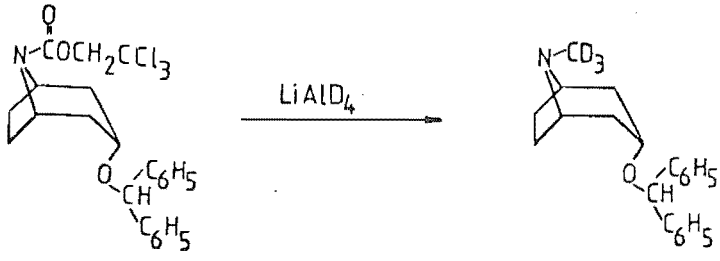


Figure 14. Preparative methods for D_0- and D_3-benztropine

thin layer chromatography. When reaction was complete the mixture was quenched and workup gave N-trideuteriomethyl-norbenztropine in 95% yield. The crude product was purified by formation of its hydrobromide which was purified by repeated recrystallisation from water. (Figure 14, scheme 3). Mass spectral analysis showed the level of D_0-benztropine contained in the purified D_3-benztropine to be less than 2.5%.

3.5 Extraction Methods for Benztropine

Benztropine has previously been extracted from plasma and urine at a pH of 9.2 using hexane[67]. The procedure included back extraction into acid, and gave an overall recovery of benztropine from doped samples of about 70%. The pH and solvent were probably selected to give an acceptable recovery of the drug from the biological matrices and yet maintain the amount of co-extracted endogenous material at a reasonable level. In this work 1-chlorobutane was selected as the solvent to extract benztropine from blood and liver samples. Aged blood is often haemolysed and cannot be separated from its cellular content and analysed as plasma. 1-Chlorobutane has been widely used to extract a number of drugs from biological samples[78-83], is noted for having efficient and clean extraction characteristics and is used by the local Government laboratory in its toxicological drug screening procedures. Hexane was rejected as an extraction solvent because it frequently gave emulsions and a lower drug recovery.

Post-mortem Blood

Haemolysed blood was extracted as 1 mL aliquots in 10 mL glass tubes with ground glass stoppers. The blood was made basic with sodium hydroxide and extracted twice with 1-chlorobutane. The combined organic phase was reduced in volume and extracted into acid. After washing with the extraction solvent the aqueous phase was made alkaline and re-extracted with 1-chlorobutane. Several drops of a 1% solution of hydrochloric acid in methanol were added and the organic solvent was evaporated. The extracts were reconstituted in ethanol and analysed by ammonia CI GCMS.

The recovery of benztropine from blood using this extraction procedure, shown in Table 18, was determined using GCMS by spiking drug-free blood with benztropine at the concentrations shown, extracting the blood and adding D_3-benztropine prior to evaporation of the organic solvent.

Post-mortem Liver.

Drugs in tissue samples must be released from their encapsulating environment prior to extraction. Methods used to destroy tissues rely upon the removal of proteins by protein hydrolysis or protein precipitation. Protein hydrolysis involves treating tissue homogenates with strong acid or alkali[84], or with a proteolytic enzyme[85]. Protein precipitation involves the addition of a reagent, such as tungstic acid, to denature protein molecules[86]. In this work homogenised human post-mortem liver tissue was buffered at a suitable pH and incubated with the bacterial proteolytic enzyme subtilisin Carlsberge to hydrolyse tissue proteins. An aliquot of the liver digest was then extracted

Blood benztropine concn.	% Recovery (mean of 5)	Range
50 ng mL^{-1}	76	68-85
200 ng mL^{-1}	78	73-82

Table 18. Recovery of benztropine from post-mortem blood.

Liver benztropine concn.	% Recovery (mean of 5)	Range
250 ng g^{-1}	72	65-81
1 µg	74	66-88

Table 19. Recovery of benztropine from post-mortem liver (drug added to liver digest)

using the procedure described for blood and plasma, and analysed by GCMS. Subtilisin Carlsberge has been used previously to digest human liver tissues and 1-chlorobutane has been reported to give clean and emulsion-free extracts (determined using a nitrogen-phosphorus detector) of the resulting digest[87]. Enzyme hydrolysis avoids the disadvantages of drug degradation or drug coprecipitation common with the more traditional pre-extraction procedures used for drug analysis in tissues. Subtilisin Carlsberge cleaves most peptide bonds in proteins over a broad range of pH (7.0-11.0) and exhibits maximum proteolytic activity at lower temperatures than normally required for acid or alkaline hydrolysis.

The recovery of benztropine from post-mortem liver, determined in a similar manner to blood, is shown in Table 19. The recovery of benztropine extracted from both post-mortem blood and liver shows a variability that would be unacceptable in a quantitative assay using external standards. This variability presents no difficulties in the assay described here, however, because an internal standard is used which accommodates this.

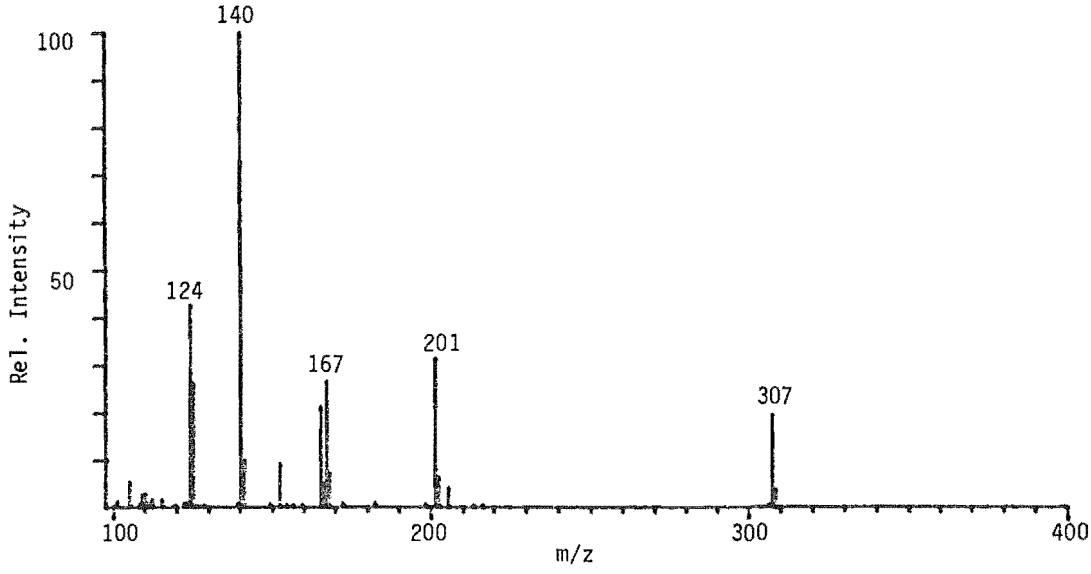
3.6 Gas chromatography

Benztropine is thermally stable and sufficiently volatile to be chromatographed without derivatisation.

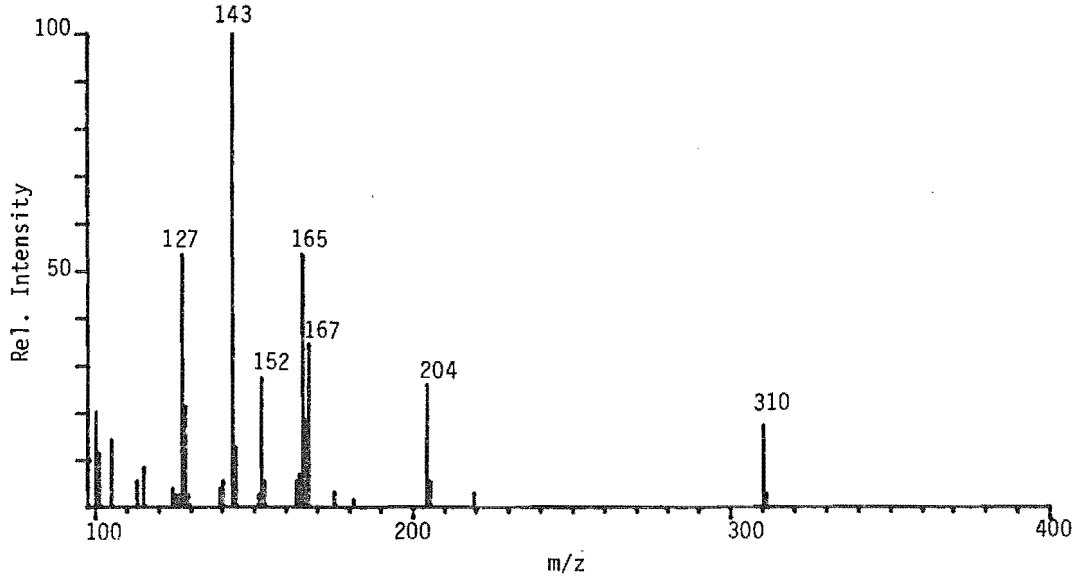
3.7 Mass Spectrometry of Benztropine

The EI mass spectra, and methane, isobutane and ammonia CI mass spectra, for D_0-benztropine and D_3-benztropine were obtained and are shown in Figures 15 and 16. The EI spectra

Figure 15. EI mass spectra of D_0- and D_3-benztropine.

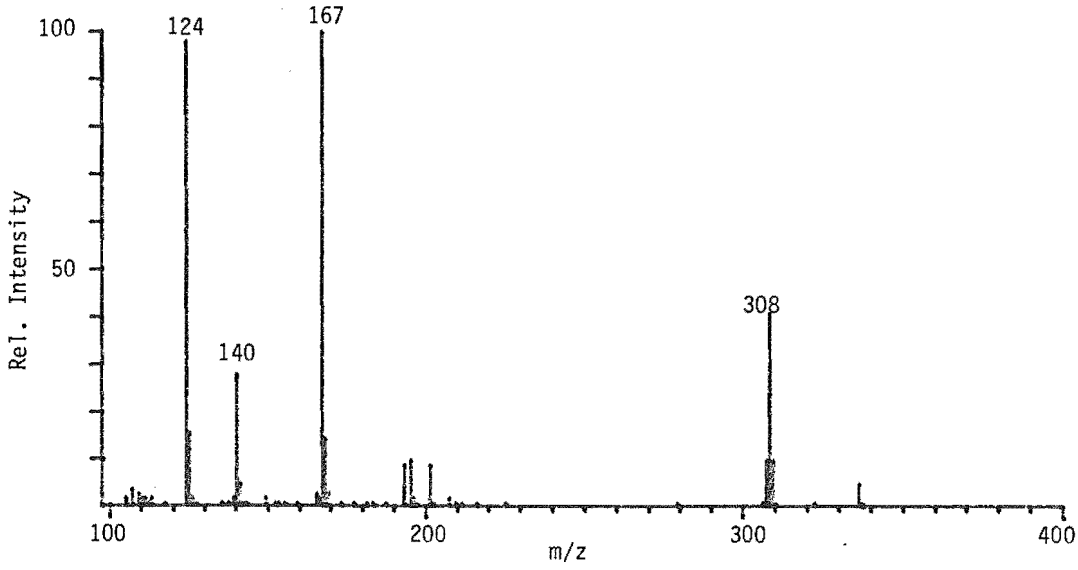


(a) EI mass spectrum of D_0-benztropine

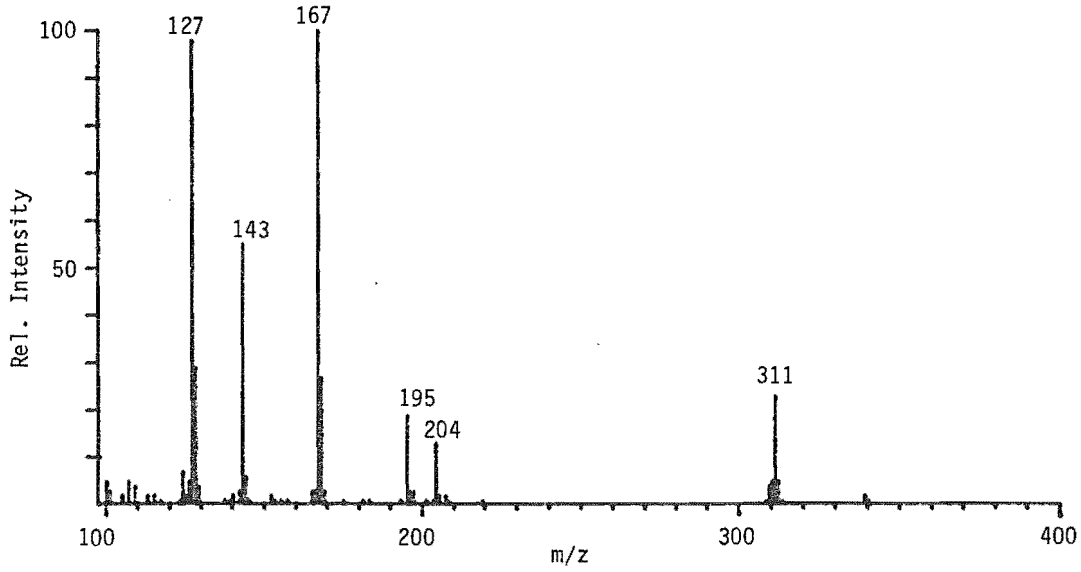


(b) EI mass spectrum of D_3-benztropine

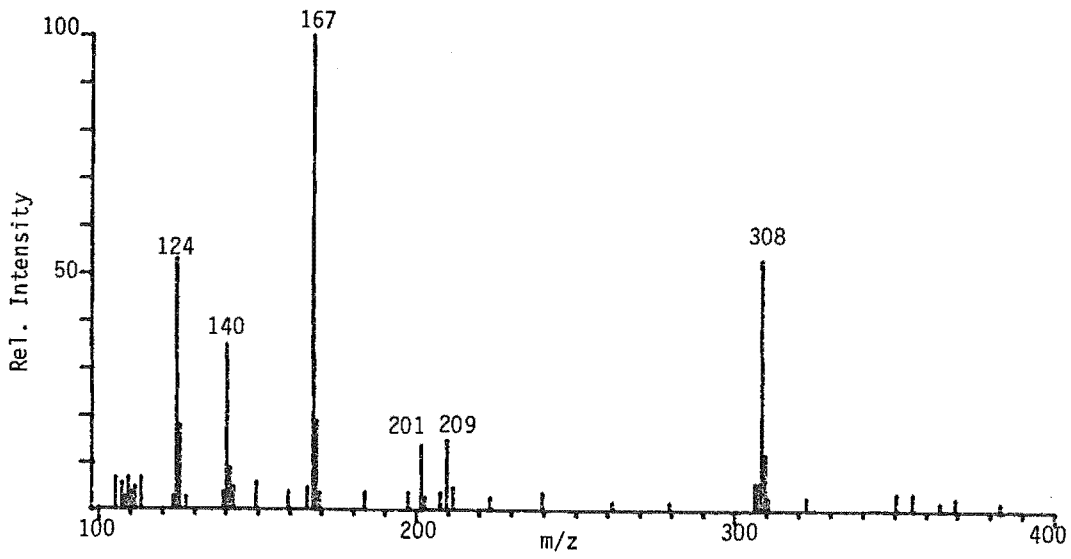
Figure 16. CI mass spectra of D_0- and D_3-benztropine using CH_4, C_4H_{10} and NH_3 as reagent gases.



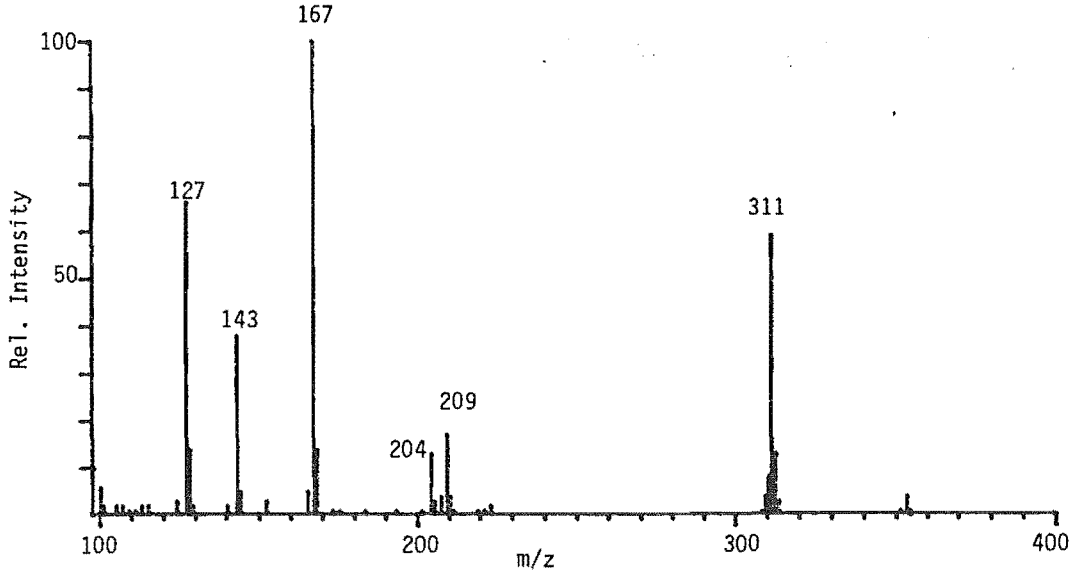
(a) CI(CH_4) mass spectrum of D_0-benztropine



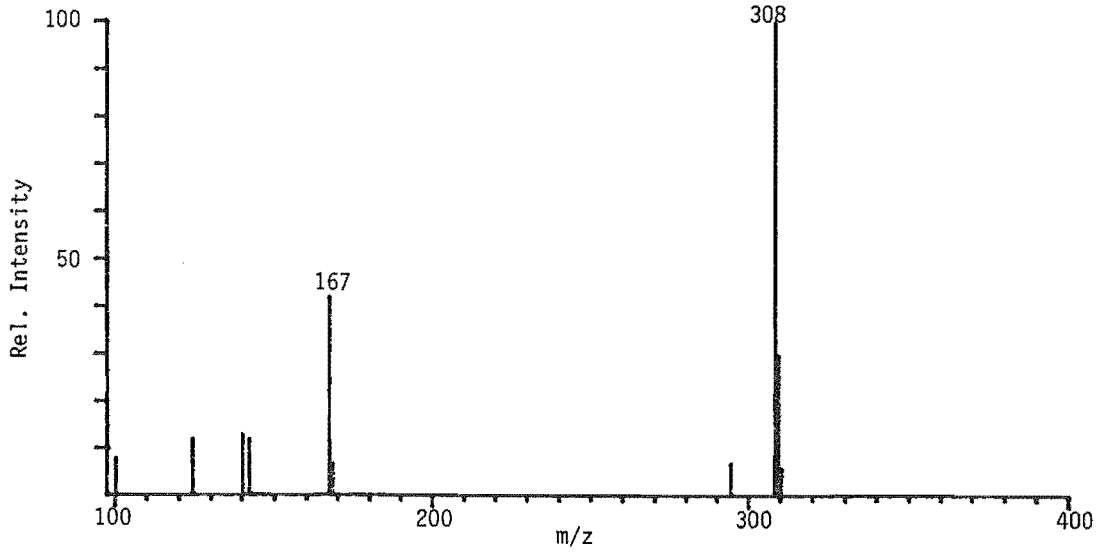
(b) CI(CH_4) mass spectrum of D_3-benztropine



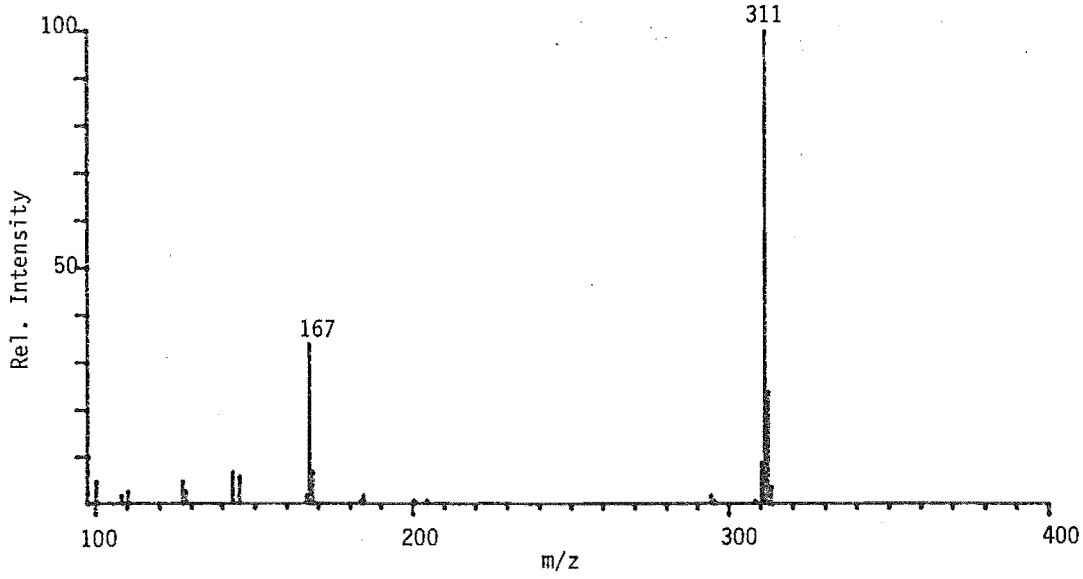
(c) CI(C_4H_{10}) mass spectrum of D_0-benztropine



(d) CI(C_4H_{10}) mass spectrum of D_3-benztropine



(e) CI(NH_3) mass spectrum of D_0-benztropine



(f) CI(NH_3) mass spectrum of D_3-benztropine

show molecular ion peaks (M^+) (m/z 307 and 310), and extensive fragmentation with base peaks m/z 140 and 143 respectively. The CI spectra show protonated molecular ion peaks (MH^+) (m/z 308 and 311), and much less fragmentation. The base peak with ammonia reagent gas is MH^+ but with methane and isobutane it is a fragment ion. Both compounds show less fragmentation with ammonia than with methane or isobutane. A large fraction of the total ion current is carried by MH^+ - methane (7%); isobutane (12%) and ammonia (43%). This suggested that monitoring MH^+ under CI, particularly with ammonia reagent gas, might provide high sensitivity for the quantitation of benztropine. As was the case with the CI spectra of TMS_2-morphine, the CI spectra all show sufficient fragmentation to allow high specificity to be attained by monitoring more than one ion.

A comparison of sensitivities was made experimentally by injecting equal amounts of benztropine into the GCMS while the mass spectrometer was monitoring the base peak under EI (m/z 140) and then m/z 308 using CI for all three reagent gases. Ion intensities were determined and compared by measuring peak heights on a chart recorder. The CI response for MH^+ with ammonia was about one and a half times that obtained with isobutane, which in turn was about the same as that obtained with methane. The CI response with all three reagent gases was greater than that obtained while monitoring the base peak under EI. CI sensitivities were also compared for the base peak of benztropine with methane (m/z 167), isobutane (m/z 167) and ammonia (m/z 308), and again the response was greatest with ammonia. Based on these results, ammonia CI was selected for the

quantitation of benztropine in blood extracts and the detection of benztropine in liver extracts.

3.8 GCMS Analysis

Quantitation of benztropine in blood was based on the MH^+ ion intensities from D_0-benztropine (m/z 308) and D_3-benztropine (m/z 311). The procedures used were similar to those which have been described earlier for the quantitation of morphine in plasma. The GC column and the ion source were saturated with D_0- and D_3- benztropine before beginning analysis of the blood extracts. Figure 17 shows selected-ion chromatograms (m/z 308 and 311) near the retention time for benztropine for extracts prepared from doped blood and for extracts prepared from blank blood. A calibration graph was prepared to establish that the relationship between the mass spectrometer ratios and the mass ratios was linear. The calibration graph was prepared using extracted blood that contained D_0- and D_3-benztropine in the ratios 0.25, 0.50, 1.0 and 2.0 D_0:D_3 with $D_0 + D_3$ = 100 ng mL^{-1}. The preparation of the standard mixtures of D_0- and D_3-benztropine used to spike the blood is discussed in the experimental section. A weighted linear least squares procedure was used to find the regression line for the calibration graph, in a similar manner to that which has been described for morphine in the previous section of this thesis. The ion intensity ratios and statistical analysis are given in Table 20, and the calibration graph is presented in Figure 18. The data affirm a simple linear relationship between the appropriate ion intensity ratios and the concentration of benztropine in blood. The accuracy

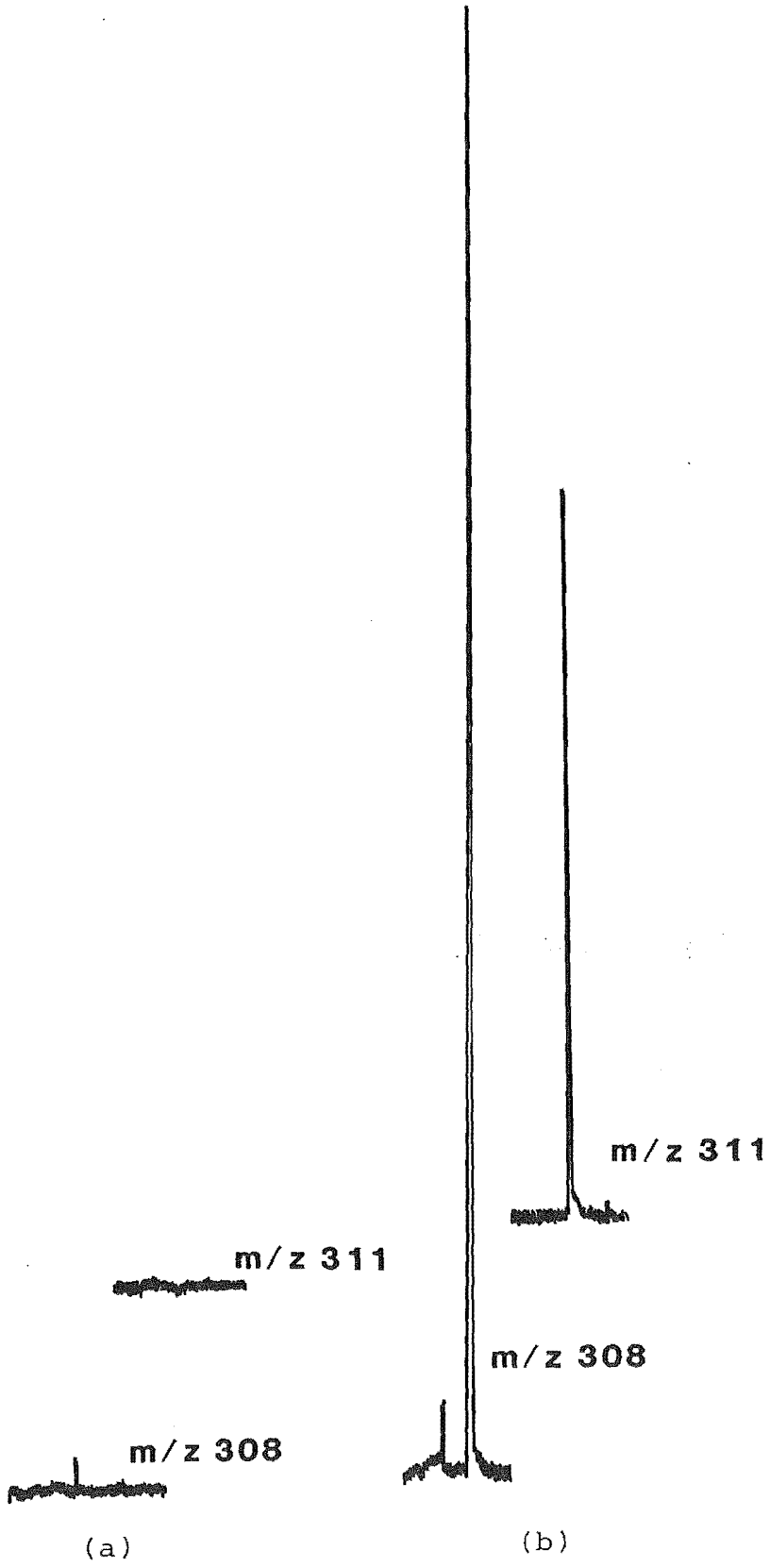


Figure 17. Response at m/z 308 and 311 near the retention time for benztropine for (a) an extract from blank blood and (b) an extract from blood doped with $D_0 + D_3$ = 100 ng mL^{-1} benztropine.

TABLE 20. CALIBRATION DATA AND REGRESSION ANALYSIS STATISTICS

Concn. in blood ($D_0 + D_3$)	D_0/D_3 ratio	n	$\bar{x}$	s
100 ng mL^{-1}	0.25	5	0.264	0.02904
"	0.50	"	0.532	0.04628
"	1.00	"	1.031	0.06283
"	2.00	"	1.943	0.17388

Correlation coefficient (r)	Gradient	% of explained variation $100\ r^2$	Parameters for least squares line $y = a + bx$	Theoretical value of r at 99.9%
0.9992	0.9965	99.8	$y = 0.02088 + 0.9965x$	0.6787

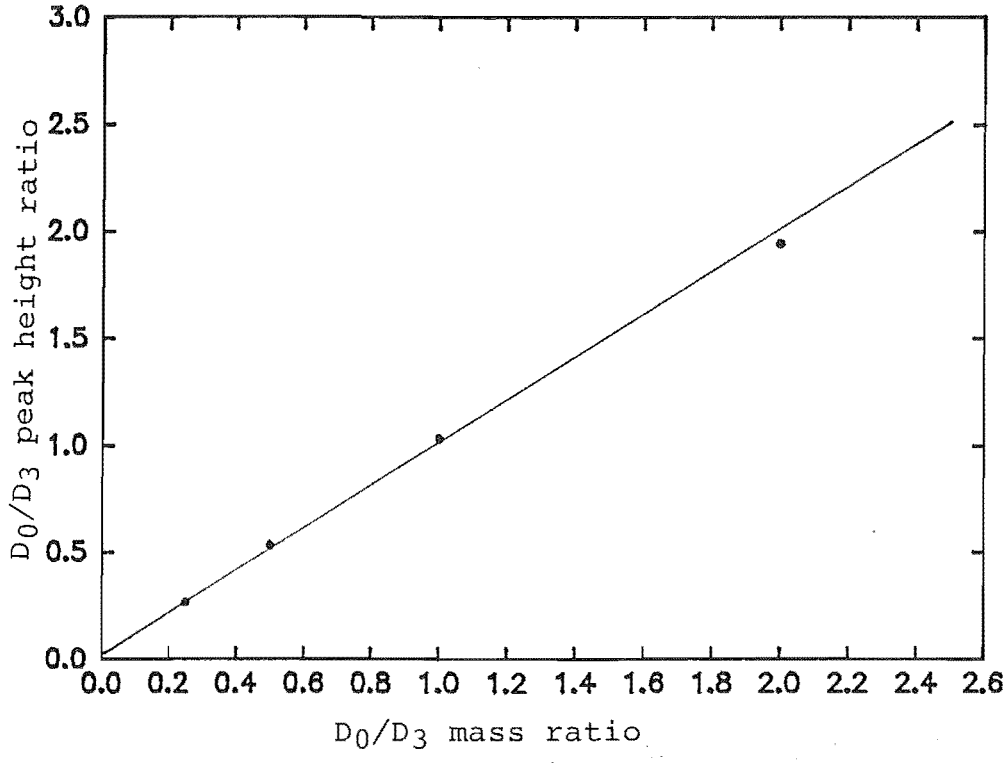


Figure 18. Calibration graph for benztropine in blood (D_0 + D_3 = 100 ng mL^{-1}, ● = mean of 5 measurements)

of the procedure for measuring benztropine was assessed by six determinations of a single doped blood sample containing benztropine at a concentration of 50 ng mL^{-1}. Measured levels were 47.4 ± 3.6 ng mL^{-1} when $D_0:D_3 = 1$, and 46.2 ± 6.3 ng mL^{-1} when $D_0:D_3 = 0.5$.

Figure 19 shows selected-ion chromatograms (m/z 308 and 311) near the retention time for benztropine for extracts prepared from doped and blank liver samples. The doped sample contained benztropine at a concentration of 50 ng $gram^{-1}$ of liver. The traces are free of interference at both m/z values, and suggest that D_3-benztropine could also be used for the quantitation of benztropine in liver samples.

3.9 Interference by other Drugs

A list of drugs with which benztropine is commonly coadministered is presented in Table 21[88]. These drugs may be present in biological specimens presented for analysis of their benztropine concentration. To determine if any of these drugs would interfere with the detection of benztropine in blood and liver samples analysed by the present method, a blood and a liver sample were spiked with each of the drugs listed in Table 21 at a concentration of 10 μg mL^{-1} and 50 μg $gram^{-1}$ respectively, and then extracted and analysed in the manner which has been described. No peaks were detected near the retention time of benztropine while monitoring MH^+ ions from D_0- and D_3- benztropine.

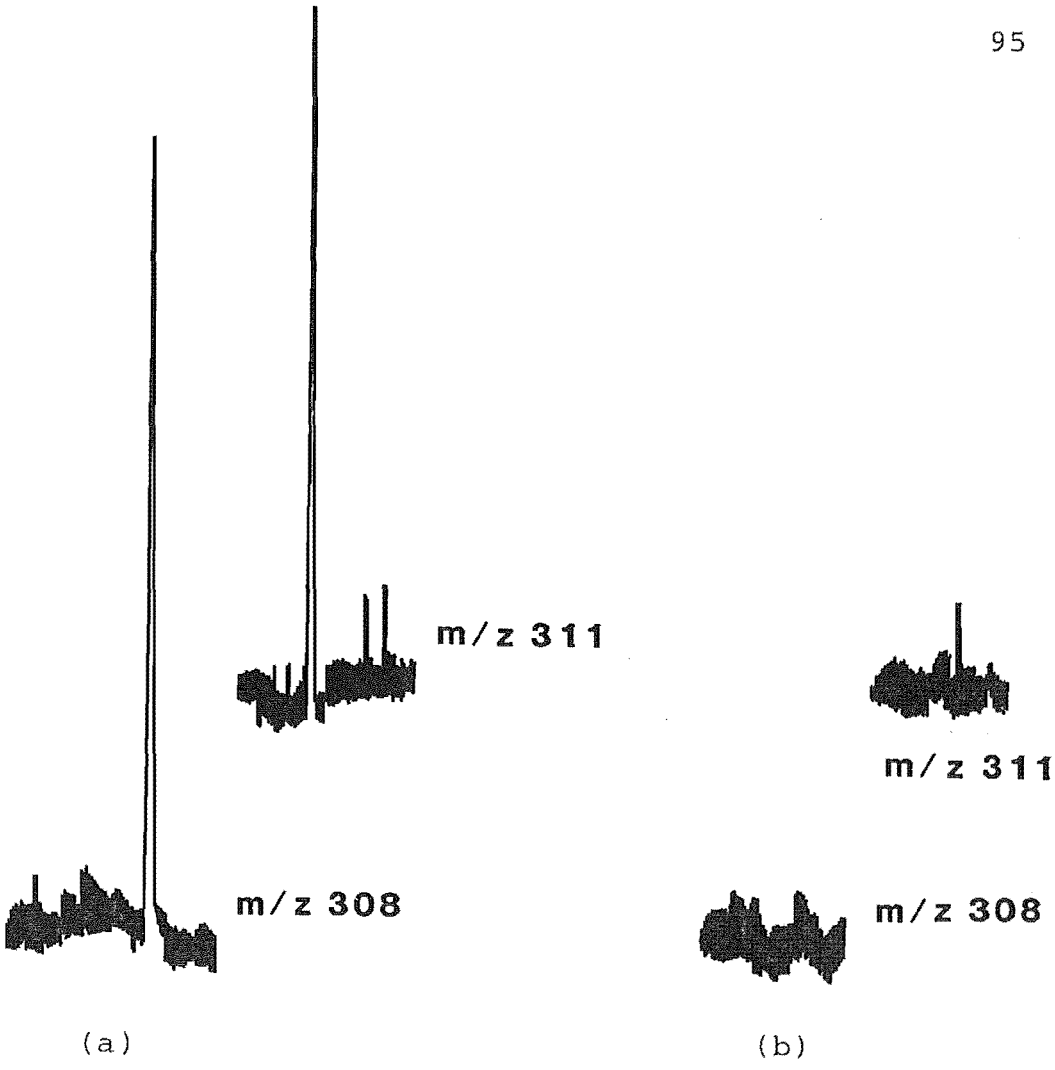


Figure 19. Response at m/z 308 and 311 near the retention time for benztropine for (a) an extract from liver doped with $D_0 + D_3$ = 100 ng $gram^{-1}$ benztropine, and (b) an extract from blank liver.

Triflupromazine

Haloperidol

Chlorpromazine

Triazolam

Lorazepam

Lithium carbonate

Table 21. Drugs commonly coadministered with benztropine

3.10 Interpretation of Results

The CI GCMS assay presented here is sensitive and specific for measuring benztropine concentrations in post-mortem blood, and for detecting benztropine in post-mortem liver. The assay is more specific and potentially more sensitive than the GCMS assay using EI ionisation which has previously been reported[67]. The absolute sensitivity obtainable depends on a number of factors including the volume (or mass) of sample which is prepared for analysis, and the amount of material which is injected onto the gas chromatographic column for detection by the mass spectrometer. In this work 1 mL blood samples and 10 gram liver samples were extracted, but this volume and mass could be increased to give a corresponding decrease in the assay's detection limit. The detection limit could be lowered still further by using an on-column injector on the gas chromatograph - the on-column injector used during the work described in Chapter 2 of this thesis was not available when the present assay was developed. In its present form a detection limit for benztropine of 2 ng mL^{-1} in blood and 10 ng $gram^{-1}$ in liver seems possible.

The extraction procedure described is adequate for handling partially decomposed blood and liver samples. A good recovery of benztropine was obtained and the amount of coextracted endogeneous material was minimal, as indicated by a lack of any significant background ions when the total ion current for a blood and a liver sample containing no benztropine was monitored.

Other drugs commonly coadministered with benztropine were shown not to interfere with this assay. Blood and

tissue samples (liver in particular) are likely to contain metabolites of these drugs, however, and further work is required to determine if the chromatographic conditions used here need to be modified to eliminate interference by these substances.

Conclusion

The procedure described here is highly specific and sensitive for detecting and measuring benztropine in post-mortem blood, and for detecting benztropine in liver.

CHAPTER 4

INTRODUCTION

4.1 Datura Stramonium

Datura stramonium, also called Thorn Apple, Jimson weed and Jamestown weed, is a poisonous plant that grows in both waste and cultivated land in various parts of New Zealand. The plant is indigenous to the region of the Caspian Sea and is one of fifteen to twenty species of Datura which range from annual and perennial herbs to shrubs and trees. These plants are all members of the Nightshade (Solanaceae) family[89,90].

All parts of the D. stramonium plant (leaves, flowers, seeds and roots) contain the physiologically active tropane alkaloids (-)-hyoscyamine and (-)-scopolamine (hyoscine). The racemic form of (-)-hyoscyamine is atropine and this alkaloid may also be present in parts of the plant. Hyoscyamine and (-)-scopolamine are esters of tropic acid with the bases tropine and scopoline respectively. The structures of atropine and scopolamine have been presented in Figure 13.

4.2 Poisoning by Datura Stramonium

Atropine and scopolamine act on the body by preventing the neural transmitter acetylcholine from exerting its usual effect on receptor sites at nerve junctions. Symptoms of poisoning by these compounds are characterised by nausea and vomiting, a dry mouth, dilated pupils and hallucinations and fever. Accidental poisoning following ingestion of D. stramonium is uncommon, both in humans and in livestock, in

this country and overseas. Accidental poisonings have mainly involved young children eating the plant seeds[91-95], which have a high alkaloid content. In recent years there has been an increasing incidence of intoxication and poisoning following abuse of this plant. This is largely the result of the discovery that the leaves of D. stramonium can yield a legal and readily obtainable hallucinogen. Reported cases of abuse have usually involved the drinking of an aqueous plant extract, referred to as "Datura tea". The extent of the (often prolonged) effects of drinking such a preparation are illustrated by a recent newspaper report of three youths who were found experimenting with the plant[96]:

"The teenagers were hallucinating some hours after they were brought into the (police) station and one of them, who had been released because he seemed better, was found upstairs at the station taking his socks off and saying he was going to bed. Another was convinced he was fixing the carburettor of his car. They were away with the fairies....."

4.3 This work

There are numerous references in the literature concerning the alkaloid content of D. stramonium, but nothing is known about the alkaloid content of aqueous extracts prepared from the leaves. The chemical properties of atropine[97], the main alkaloid in D. stramonium leaves, suggest that such extracts might contain high levels of this compound.

This section of the thesis describes a brief investigation to determine how efficiently atropine is extracted into water when leaves of D. stramonium are used to prepare a "Datura tea". The atropine concentration in an aqueous extract, and in an extract prepared by exhaustive organic solvent extraction of plant leaves was measured in a CI QSIM assay for atropine using trideuterionoratropine as an internal standard. The study was prompted by a request from a nearby Government Laboratory for information about atropine concentrations in aqueous D. stramonium extracts. The Laboratory has recently experienced difficulty in detecting atropine in post-mortem specimens taken from people who have died from a drug overdose and who may also have been drinking Datura tea at the time of their death.

RESULTS AND DISCUSSION

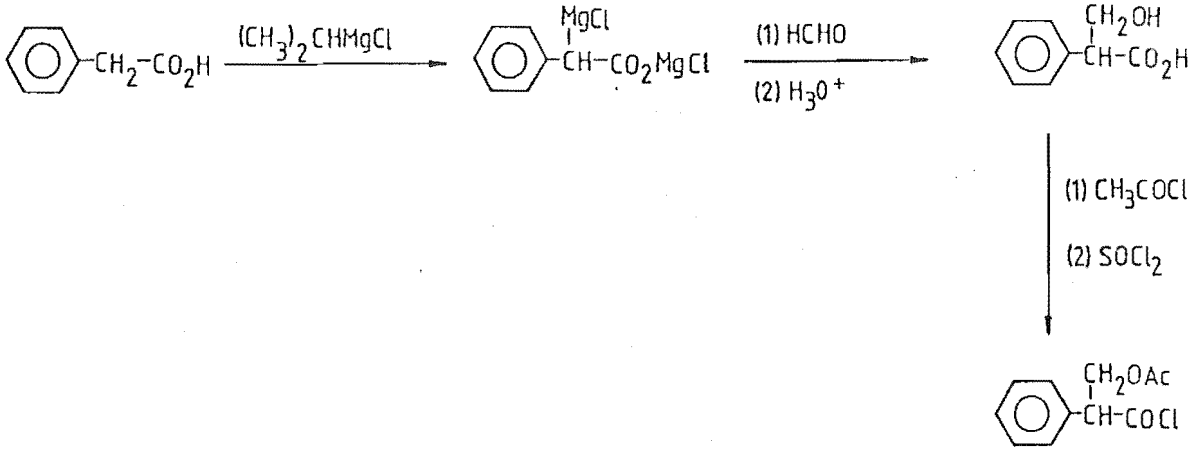
4.4 Discussion of Preparative Procedures

N-Trideuteriomethylnoratropine (D_3-atropine) was prepared from N-trideuteriomethylnortropine and acetyltropylchloride using the method of Fung and DeGraw[98]. Addition of formaldehyde to the Grignard derivative of phenylacetic acid, gave after hydrolysis, tropic acid[99]. This was treated with acetyl-chloride and then thionyl chloride to give acetyltropyl-chloride[100] (Figure 20, scheme 1). N-Trideuteriomethylnor-tropine was prepared by the lithium aluminium deuteride reduction of N-ethoxycarbonylnortropine which was formed by reaction of tropine with ethylchloroformate[98] (Figure 20, scheme 2). Addition of acetyltropylchloride to N-trideuterio-methylnortropine with heating gave, after acid hydrolysis and workup, N-trideuteriomethylnoratropine which was purified by repeated recrystallisation from acetone (Figure 20, scheme 3). Mass spectral analysis showed the level of D_0-atropine contained in the purified D_3-atropine to be less than 1%.

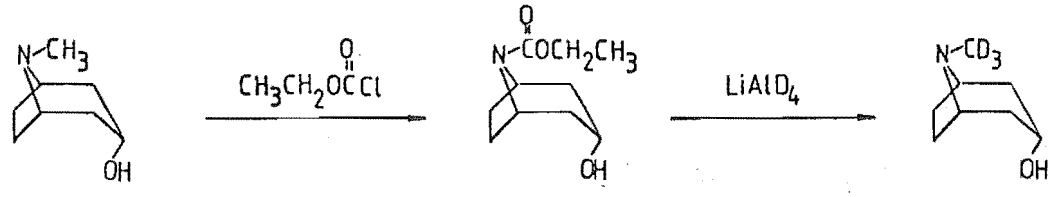
4.5 Plant Material

Seeds of *D. stramonium* were obtained from the Botany Division of the Department of Scientific and Industrial Research. The seeds were raised in the University's Botany Department glasshouse and several young plants were transferred to the University nursery. Leaves of the plants were collected when the plants were in the flowering-fruiting stage of development, this having been

Scheme 1:



Scheme 2:



Scheme 3:

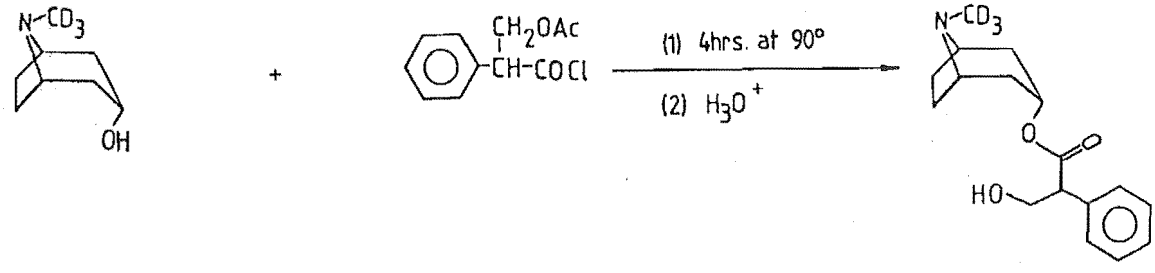


Figure 20. Preparative methods for D_3-atropine

reported as the time of maximum alkaloid content. The leaves were air dried and stored in air tight containers.

4.6 Preparation of an Aqueous Plant Extract

Unsuccessful attempts were made to obtain information about how Datura tea is usually prepared. Several people who had been apprehended after drinking aqueous Datura extracts were approached (by the Police) but were unwilling, or unable, to give this information.

One book, describing the history, cultivation and use of garden herbs gives the following information concerning herb teas[101]:

"They are made with an infusion of fresh or dried leaves or flowers and boiling water, and left to steep for a few minutes with a lid on the pot or a cover over the cup, and drunk either hot or cold.... the amount to use when making a herb drink is a tablespoon of fresh leaves to one pint of water, and half that amount of herbs, or even less, if using dried leaves."

Based on this recipe, dried D. stramonium leaves (2.50 g) were soaked in boiling water (1140 mL) in a covered glass beaker for several minutes. The aqueous extract was made up to a volume of 2 L with water and four 1 mL aliquots of this extract were taken. To three of these samples was added an appropriate amount of D_3-atropine. All samples were then made basic with concentrated ammonia solution and extracted with chloroform in 10 mL glass tubes with ground glass stoppers. The extracts were reconstituted in ethanol and kept cold until analysed.

4.7 Soxhlet Extraction of Plant Material

The procedure for extracting alkaloids from the plant material was based on a previously reported method[102]. Dried *D. stramonium* leaves (2.50 g) were exhaustively extracted in a soxhlet apparatus with a mixture of ammonia, ethanol and ether (total volume 1140 mL). The organic extract was made up to a volume of 2 L with additional ether and three 1 mL aliquots of the extract were taken. To each was added an appropriate amount of D_3-atropine. The extracts were then taken up in acid, made basic with concentrated ammonia solution and finally extracted into chloroform as for the aqueous extracts.

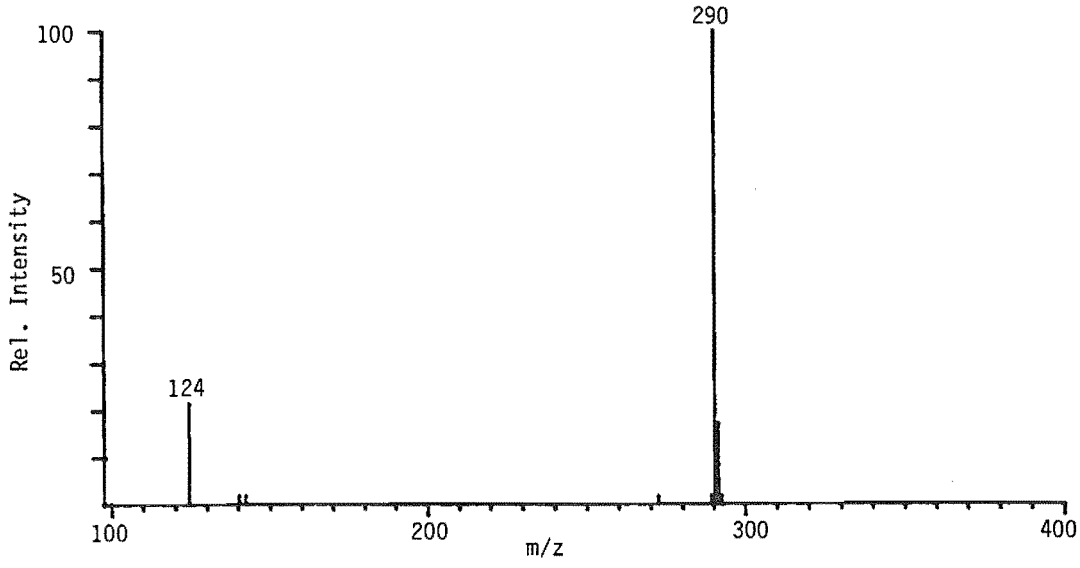
4.8 Gas Chromatography of Atropine and Scopolamine

Both atropine and scopolamine can be chromatographed wihout derivatisation. A potential difficulty with the analysis of atropine and scopolamine by gas chromatography is the dehydration of these compounds in the heated injector port, and on the GC column, to form apoatropine and aposcopolamine respectively[103]. This did not present a problem for the quantitation of atropine in this work because D_3-atropine was used as an internal standard.

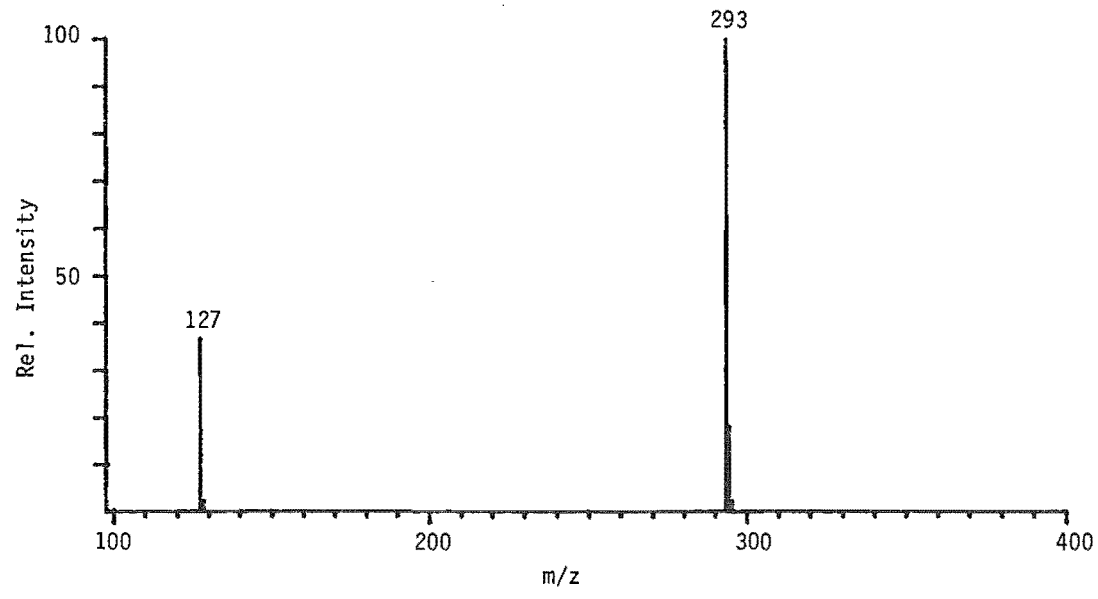
4.9 Mass Spectrometry of Atropine and Scopolamine

The EI mass spectra of both atropine and scopolamine have previously been reported[104] and will not be presented here. The ammonia CI mass spectrum of D_0- and D_3-atropine were recorded and are presented in Figure 21. The CI spectra show protonated molecular ion peaks (MH^+) (m/z 290 and 293) and no significant fragment ions.

Figure 21. CI(NH_3) mass spectra of D_0- and D_3-atropine.



(a) CI(NH_3) mass spectrum of D_0-atropine



(b) CI(NH_3) mass spectrum of D_3-atropine

4.10 Analysis of Plant Material

A total ion current chromatogram of the aqueous plant extract to which no D_3-atropine had been added was recorded with the mass spectrometer operating under EI conditions. Two peaks were seen which were identified from their mass spectrum as atropine and scopolamine. The major component in the extract was atropine, as expected. The scopolamine concentration was estimated to be twenty to twenty-five times lower than the atropine concentration, based on measurement of peak heights. Quantitative analysis for atropine in both the aqueous and soxhlet extracts was performed using GCMS with the mass spectrometer operating under CI with ammonia reagent gas. Quantitation was based on measuring the MH^+ ion intensities of D_0- and D_3-atropine. A calibration graph was constructed using standard solutions that contained D_0- and D_3-atropine in the ratios 0.25, 0.5, 1.0, and 2.0 D_0:D_3 with $D_0 + D_3 = 10$ ng μL^{-1}. The preparation of these standard mixtures is discussed in the experimental section. A weighted linear least squares procedure was used to find the regression line for the calibration graph, in a similar manner to that which has been described for morphine and benztropine in earlier sections of this thesis. The measured ion intensity ratios for the calibration standards and associated statistical analysis, and the resulting calibration graph are presented in Table 22 and Figure 22 respectively. The measured ion intensity ratios for the aqueous and soxhlet plant extracts, and the associated calculations to determine the atropine concentrations in these extracts are presented in Table 23.

TABLE 22. CALIBRATION DATA AND REGRESSION ANALYSIS STATISTICS

Concn. of injection ($D_0 + D_3$)	D_0/D_3 ratio	n	$\bar{x}$	s
10 ng μL^{-1}	0.25	3	0.257	0.01527
"	0.50	"	0.523	0.06028
"	1.00	"	1.034	0.04163
"	2.00	"	2.023	0.08145

Correlation coefficient (r)	Gradient	% of explained variation	Parameters for least squares line	Theoretical value of r at 99.9%
0.9999	1.0199	99.8	y = 0.0034 + 1.0199x	0.8233

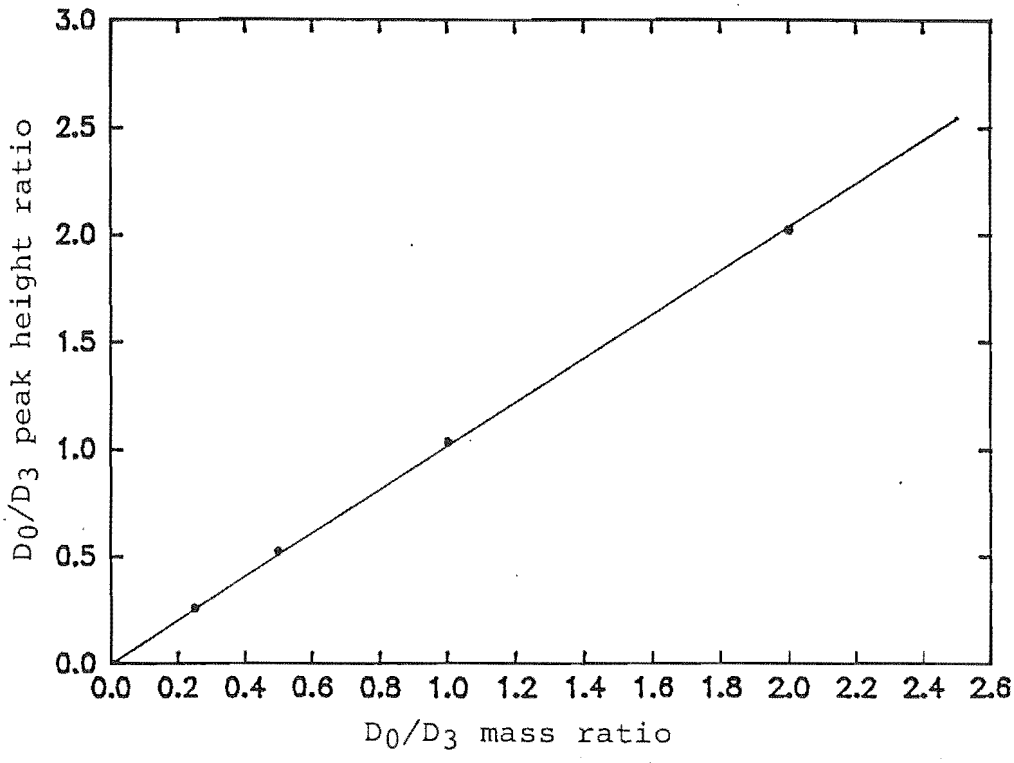


Figure 22. Calibration graph for atropine determination (D_0 + D_3 = 10 ng μL^{-1}, ● = mean of 3 measurements)

TABLE 23. RESULTS FOR ATROPINE CONCENTRATIONS IN PLANT EXTRACTS

AQUEOUS EXTRACT

Sample No.	D_3-atropine concn. ng μL^{-1} extract	Peak height ratio	Measured atropine concn. ng μL^{-1} extract
1	5.0	0.59	2.88
2	"	0.62	3.02
3	"	0.61	2.97
			mean (±SD) = 2.96 (± 0.07)
SOXHLET EXTRACT			
1	5.0	0.66	3.22
2	"	0.63	3.07
3	"	0.65	3.17
			mean (±SD) = 3.15 (± 0.08)

4.11 Interpretation of Results

Atropine in the extract obtained by exhaustive solvent extraction of D. stramonium leaves can be assumed to represent the total (-)-hyoscyamine and atropine in the plant material (extraction of the plant material will result in racemisation of (-)-hyoscyamine to atropine). The atropine concentration measured in the soxhlet extract corresponds to a combined (-)-hyoscyamine and atropine level in the plant leaves of 0.25% by dry weight. This level is in good agreement with alkaloid levels reported in the literature for dried D. stramonium leaves[105]. The atropine concentration measured for the aqueous D. stramonium extract corresponds to a 94% recovery of available (-)-hyoscyamine and atropine. Hyoscyamine and atropine were thus very efficiently extracted from D. stramonium leaves simply by soaking the leaves in boiling water for several minutes.

A summary of the effects of atropine in relation to dosage, reproduced from the literature[106], is presented in Table 24. Wide variation in tolerance to atropine is known to exist, and what constitutes a fatal dose is unclear. Although 10 mg of atropine produces severe distress, many adults have recovered from a single dose of 100 mg and more. Recovery after ingestion of 1000 mg has been reported[107]. It is thus very difficult to make a concise statement about the dangers associated with drinking an aqueous extract prepared from D. stramonium leaves. The amount of atropine ingested will depend on the time of year that the plant material is collected, the amount of plant material and the volume of water used to prepare the extract, and the volume of extract which is consumed. From the results of this

Table 24. EFFECTS OF ATROPINE IN RELATION TO DOSAGE (Reproduced from Ref. 106)

DOSE	EFFECTS
0.5 mg	Slight cardiac slowing; some dryness of mouth: inhibition of sweating
1.0 mg	Definite dryness of mouth; thirst; acceleration of heart, sometimes preceded by slowing; mild dilation of pupil
2.0 mg	Rapid heart rate; palpitation; marked dryness of mouth; dilated pupils; some blurring of near vision
5.0 mg	All the above symptoms marked; speech disturbed; difficulty in swallowing; restlessness and fatigue; headache; dry, hot skin; difficulty in micturition; reduced intestinal peristalsis
10.0 mg and more	Above symptoms more marked; pulse rapid and weak; iris practically obliterated; vision very blurred; skin flushed, hot, dry, and scarlet; ataxia, restlessness and excitement; hallucinations and delirium; coma

study it could reasonably be expected that several medium-sized glassfuls of an aqueous extract prepared from D. stramonium leaves in the manner described in this thesis would contain sufficient atropine to constitute a dangerous drug dose.

4.12 Conclusion

An aqueous extract of D. stramonium leaves will contain most of the atropine present in the plant material. Drinking such an extract is likely to be highly dangerous.

CHAPTER 5

INTRODUCTION

5.1 Mass Spectrometry of Involatile and Thermally Labile Compounds

A major limitation in the application of the more common mass spectrometric techniques of electron impact (EI), field ionisation (FI) and chemical ionisation (CI) to the identification and structural determination of thermally unstable or involatile compounds is that the sample must be vaporised before ionisation[108,109]. Such compounds often decompose or fail to vaporise in the mass spectrometer ion source, and the resulting spectra give very little useful information. Chemical derivatisation of polar functional groups, which reduces intermolecular hydrogen bonding, has been widely used to increase the volatility and thermal stability of "difficult" samples[110]. Unfortunately derivatisation adds an extra step to sample analysis, and also gives an increase in molecular weight which is proportional to the number of polar groups present.

In recent years a number of techniques have been developed which allow many non-derivatised thermally unstable and involatile compounds to be investigated almost routinely by mass spectrometry[111-115]. These techniques have used a wide variety of methods to ionise sample molecules, ranging from simple heating of a metal substrate in the presence or absence of a strong electric field to impact with very high energy particles. Three of these techniques which are now commercially available are described below.

(a) Field desorption (FD)[111]

A very strong electrostatic field (1-4 V/Å) is used to ionise sample molecules absorbed on a specially prepared surface, and a combination of this field and thermal energy desorbs the ionised sample molecules. This technique was introduced by Beckey in 1969 and is probably still the most widely used method for the analysis of non-volatile samples.

(b) Plasma desorption[112]

Simultaneous ionisation and desorption of sample molecules is achieved by impact of high energy (100 MeV) fission products from ^{252}Cf onto the back side of metal foil coated with a molecular layer of the sample. This technique often gives molecular weight information when FD does not.

(c) Fast Atom Bombardment (FAB)[115]

A beam of rare gas atoms bombards the sample suspended in a relatively involatile liquid causing desorption of ions. This technique is perhaps the most important recent development in mass spectrometry and it can provide molecular weight information for very high molecular weight compounds in addition to those which are involatile and thermally fragile.

5.2 Desorption Electron Ionisation and Desorption Chemical Ionisation

Another approach to obtaining useful mass spectral information for involatile and thermally unstable compounds has been to minimise the distance within the mass spectrometer source between the site of sample

volatilisation and the site of ionisation. This strategy has led to the development of the so called "in-beam" techniques of desorption electron ionisation (DEI)[116-124] and desorption chemical ionisation (DCI)[125,126] mass spectrometry. In DEI and DCI the sample to be analysed is deposited on a surface and introduced directly inside the mass spectrometer ion source by means of an extended direct insertion probe. This replaces the usual practice of vaporising the sample outside of the source, which generally results in pyrolysis of thermally labile compounds. The sample or the ion source is then heated (after the reagent gas has been introduced for DCI). Under these conditions many involatile and thermally unstable compounds give mass spectra which exhibit abundant protonated molecular ion and fragment ion peaks which would not be observed using a conventional solids probe. For DCI spectra the extent of sample fragmentation is generally greater than that observed under FD MS so the spectra give more structural information about the sample.

The main advantage of DEI and DCI MS is its simplicity. Any mass spectrometer can be equipped with a direct exposure probe without major modification, and at relatively low cost. By comparison, FD, plasma desorption and FAB methods are expensive and are usually only available on larger magnetic sector instruments.

Two approaches have been taken in an effort to enhance the volatility of thermally labile molecules deposited on the tip of an extended probe - the use of inert surfaces and rapid probe heating. Dispersal of molecules onto a relatively inert surface reduces the binding energy of the

sample to the surface and makes the desorption process more competitive with decomposition. Materials used as vaporisation surfaces have included glass[125,127], quartz[117,128-130], Teflon[119,131,132], Vespel[133,134], bare and polyimide coated wires[124,135-138], and polyimide-coated fused silica capillary tubing[139]. The rapid heating technique is based on a kinetic analysis of the competitive decomposition and evaporation processes. It has been shown[119,120] that rapid heating of the probe tip favours the desorption of intact neutral molecules over decomposition. For this reason heating rates of $10^{o}C$ per second (termed "flash desorption") have been used. A consequence of rapid sample heating is that desorption occurs very quickly so scanning of the mass spectrum must be very rapid. Computer data acquisition is usually necessary (alternatively, simultaneous detection of all ions formed using a photographic plate is possible with a mass spectrometer of Mattauch-Herzog geometry[124]).

The mechanism of the ionisation process occurring at the tip of the extended probe during DEI and DCI is still uncertain. Rapid heating of the sample on an inert surface presupposes the process to involve desorption of neutral sample molecules from the surface followed by their gas-phase ionisation by the electron beam (in DEI) or by the reagent gas plasma (DCI)[134]. Direct ionisation of the sample on the probe surface followed by desorption of ions (i.e. a heterogeneous reaction), has also been suggested as a mechanism[132], and experimental support for both hypotheses has been presented. A review of the evidence[140] has concluded that in most reported DEI and DCI experiments the

process has been one of short path thermal volatilisation followed by gas phase ionisation.

Spectra from DEI and DCI experiments showing molecular ions usually appear immediately after insertion of the extended probe, and the spectra invariably exhibit protonated molecular ions (MH^+). Later spectra under DEI begin to look like conventional EI spectra. The formation of MH^+ ions during DEI has been attributed to the rapid rise in the ion source pressure as molecules are desorbed from the probe surface, temporarily causing chemical ionisation conditions. Most experiments have been conducted essentially without regard to quantitative sample measurements, and most spectra have been obtained from sample sizes of 100 ng to a few micrograms. It appears that the sensitivity of DCI is comparable to that of ordinary CI.

To date, most DEI- and DCI-MS studies have been concerned with developing the in-beam technique, and demonstrating its ability to provide molecular weight and structural information for involatile and thermally unstable compounds. Some classes of compound for which in-beam spectra have been reported are listed in Table 25. The following examples, taken from this list, illustrate the potential of DEI- and DCI-MS.

Amino acids:

Most amino acids show molecular ion peaks in their EI, CI and FI mass spectra[109,145-148]. Creatine and arginine are exceptions, probably because both compounds undergo thermal dehydration to form the corresponding cyclic lactones at temperatures below that required for

volatilisation[146,148]. The DCI mass spectra obtained with methane[126], isopentane[132] and isobutane[133] as reagent gas show prominent MH^+ ion peaks for both the parent compounds and the dehydration products.

Carbohydrates:

The ability to analyse carbohydrates by mass spectrometry is severely limited by the thermal instability and extremely low volatility of these compounds[124]. DEI mass spectra which show prominent MH^+ ions for sucrose and lactose have been reported[124].

Polypeptides:

The low volatility of these compounds, and the small number of fragment ion peaks in their FD spectra[149], limit the utility of EI-, CI- and FD-MS for determining the sequence of amino acids present. Derivatisation can be used to increase the volatility but this can consume considerable amounts of sample[150]. DCI-MS spectra for a number of tri-, tetra-, and pentapeptides have been recorded which exhibit MH^+ ion peaks and fragmentation sufficient to derive a unique amino acid sequence[120,125,126].

5.3 This Work

The laboratory in which the work described in this thesis was undertaken operates an A.E.I. MS902 high resolution magnetic sector mass spectrometer and a Hewlett Packard 5982A GCMS. The MS902 is an EI-only instrument, and the HP5982 provides EI and CI. Both instruments are used for research projects in toxicology, pharmacology and

environmental analysis and to provide routine mass spectral information for a number of research groups in other fields, both within and external to the University. Neither instrument has been able to provide satisfactory mass spectra for the increasing number of unstable or high molecular weight compounds being submitted, particularly those from the large marine chemistry group. This section of the thesis describes modifications made to the Hewlett Packard instrument to allow it to be used in DEI or DCI mode with a commercially available DCI probe. The effectiveness of these modifications is discussed.

Table 25 Classes of compound previously studied by DEI- and/or DCI-MS.

1.	Glucuronides	141,142,138,139
2.	Carbohydrates	124,138,139
3.	Steroids	143,142,138
4.	Prostaglandins	142,138,139
5.	Nucleotides	126
6.	Amino acids	126,132,133
7.	Nucleic acids	137
8.	Antibiotics	144
9.	Polypeptides	125,120,126,139
10.	Salts of organic acids	126
11.	Nucleosides	126,142
12.	Pesticides	126
13.	Glycosides	135,142,138

5.4 Probe Installation

5.4.1 General Considerations

In providing the HP5982A with DEI and DCI capability two minimum goals were set:

1. Existing parts of the instrument which required modification but which could not readily be replaced should be refabricated to incorporate the necessary changes. In this way the instrument could always be restored to its original state.

2. The existing EI and CI sensitivities of the instrument should not be significantly degraded at the expense of obtaining DEI and DCI capability.

5.4.2 The DCI Probe

The DCI probe was supplied by Vacumetrics (Vacumetrics, Ventura, Ca.) and is a modification of that described by Bruins[135]. A scale drawing of the probe is presented in Figure 37. All major parts of the probe are constructed from stainless steel. The filament is a loop of platinum wire mounted on two pins held in position by a ceramic spacer. The filament assembly (1) is mounted on the inner shaft (2) which can be slid along its axis within the outer shaft (3) by moving the probe handle (4). This movement permits the filament tip to be extended inside the ion chamber after the probe has been inserted through the solids probe insertion lock of the mass spectrometer. The maximum

possible extension of the inner shaft, and thus of the filament assembly, can be fixed by tightening a grub screw (accessible through a slot in the probe body) in a steel ring (not shown) around the inner shaft. The gap between the inner and outer shaft is sealed by the main vacuum seal assembly (5) which maintains the ion source vacuum during probe operation. The gas line (6) can be used to introduce the CI reagent gas into the ion chamber. The reagent gas exits through a hole in the ceramic feed-through (7) sweeping the filament tip. For the work described in this thesis the reagent gas was introduced into the ion chamber via the GC column inlet (Section 1.3) so the reagent gas inlet line of the probe was blocked off using a standard Swagelok connector and a short length of metal rod. The BNC connector (8) is used to deliver an electric current to the filament which is earthed to one side of the probe body.

The probe is operated as follows:
A drop of sample solution is applied to the tip of the filament using a microlitre syringe and the solvent is allowed to evaporate. The filament assembly is then retracted inside the outer shaft by moving the probe handle downwards. The probe handle is locked in the retracted position by slightly rotating the handle into a cut-out (not shown) in the probe body - this prevents the probe extending prematurely when exposed to the ion source vacuum. The probe is then inserted through the mass spectrometer's solids probe insertion lock into the ion source and the filament is moved into the ion chamber by releasing the probe handle and sliding the handle forward.

5.4.3 Solid Probe Insertion Lock

The HP5982A solid probe insertion lock has an internal diameter of only 6.35 mm which is too narrow to admit the 12.7 mm diameter outer shaft of the Vacumetrics DCI probe. This lock was replaced with a Con Vac solid probe insertion lock (Consolidated Instruments Corp., Baltimore Md). The rough-out port of the Con Vac lock will accept different internal diameter probe entrance adaptors which are screwed into position and sealed by an O-ring. A 6.35 mm adaptor was fitted to this port to admit the HP5982A direct insertion probe to the ionsource and this was replaced when appropriate with a 12.7 mm adaptor to admit the DCI probe. The distance from the ion source housing to the entry port of the new lock was identical with that for the existing lock - one section of the lock was rebuilt to achieve this. The conventional solids probe was therefore correctly positioned in the ion source with the 6.35 mm entrance adaptor. The length of the DCI probe required the 12.7 mm adaptor to be shortened from its original 76.2 mm to 46.5 mm to position the probe's outer shaft when inserted up to the hilt correctly near the ion source DIP sleeve (part 9 Figure 34).

5.4.4 Ion Source Modifications

The HP5982A dual ion source has been described in Section 1.2. The following modifications to the ion source were made to allow the tip of the DCI probe filament to enter the CI ion chamber.

1. The hole in the gold-plated heater shroud (part 1, Figure 33) through which the existing solids probe passes before entering the DIP sleeve (part 9, Figure 34) was enlarged from 15 mm to 18 mm diameter to accommodate the outer shaft of the DCI probe. The shroud was replated after enlarging the hole. It was considered undesirable to modify the existing shroud but a replacement could not be purchased or constructed. In addition to the source heater the shroud also contains two magnets which collimate the electron beam in the ion chamber. The instrument manufacturer was consulted before enlarging the shroud hole to determine whether this modification would distort the magnetic field produced by the magnets.

2. A problem inherent in the 5982A source design is the difficulty of ensuring that the probe entrance in the source block is in line with the DIP housing; this alignment changes as the copper O-ring flange seal is compressed. The original DIP sleeve was designed to guide the 6.35 mm probe into place even if the alignment was not perfect, and to seal the source ion chamber under CI conditions. The alignment problem proved to be more critical with the 12.7 mm DCI probe, because the throat of the original sleeve was too narrow to accept the end of the probe and provide the necessary alignment. The new DIP sleeve, constructed of 19 mm AF hexagonal stainless steel, had a wider bore which guided the 12.7 mm probe into position.

3. The existing plunger body (referred to as the plunger) had a 1.3 mm diameter DIP inlet hole which was too narrow to

admit the DCI probe filament tip. A new plunger body was constructed with external dimensions identical to those of the existing plunger and with a DIP inlet hole of 3.81 mm diameter. The new plunger was machined from stainless steel round bar. The fabrication of the mask for the electron entrance slit in the existing plunger could not be duplicated. The mask containing this slit for the new plunger was formed by cutting a rectangular aperture in a disc of stainless steel shim with a punch made for the purpose and fitting this disc into a stepped circle having a hole at its centre in the plunger wall. The disc was held in position with a circlip.

4. The DIP sample inlet hole in the spacer (part 15, Figure 35) was enlarged from 1.3 mm to 3.81 mm diameter to accommodate the DCI probe filament. A new spacer was not fabricated because the existing spacer could be readily replaced if necessary.

5.4.5 Initial Probe Set-up

The length of probe extension was adjusted initially with the ion source removed, to position the tip of the filament in the centre of the CI ion chamber. The DCI probe was inserted through the solids probe insertion lock up to the hilt and the filament was extended. A sight was taken down the interior of the ion source housing towards the quadrupole mass analyser and the length of probe extension was adjusted to position the tip of the filament in the centre of the ion optics axis. This position corresponds to the centre of the CI ion chamber. The grub screw in the

probe handle (Section 5.4.2) was then tightened to fix this length as the maximum possible extension of the probe.

The probe was withdrawn from the insertion lock and the ion source was replaced.

A glass guide, supplied by Vacumetrics, was used to align the filament with the probe axis on each occasion and just before the probe was inserted into the ion source. The filament is easily bent while being loaded with sample and is likely to be damaged inside the source if not aligned correctly; use of this alignment guide immediately before inserting the probe into the mass spectrometer is essential.

5.5 Evaluation of Modifications

The effect of the modifications which have just been described on the existing sensitivity of the mass spectrometer was assessed as follows:

In EI mode replicate injections of a dilute solution of methyl stearate were made into the gas chromatograph while the mass spectrometer was set to monitor the intensity of the molecular ion of this compound (m/z 298). In CI mode, with ammonia reagent gas, replicate injections of a dilute solution of benztropine (Section 3.1) were made while monitoring the $(M+1)^+$ ion (m/z 308). In both cases the ion intensities, measured as peak heights on a chart recorder, were averaged and compared with ion intensities measured under identical conditions before the ion source modifications were made.

The results showed that the instrument modifications caused no significant change in sensitivity in either EI or CI mode.

The first sample to be examined using the DCI probe was the amino acid creatine. This compound has previously been selected for use in optimising experimental parameters during DCI experiments[132]. A 1 μL aliquot of a freshly prepared aqueous solution of creatine (500 ng μL^{-1}) was applied to the probe filament by the method described earlier. The probe was inserted into the mass spectrometer operating under CI conditions with methane reagent gas. The probe tip was extended to the centre of the ion source and then rapidly heated by passing a current of 2.9A through the filament. Spectra were recorded repetitively at the maximum scan rate of 325 amu/second immediately after probe insertion. The "best" spectrum of creatine obtained is presented in Figure 23. It shows a protonated molecular ion peak $(M+1)^+$ (m/z 132), and a base peak corresponding to the protonated cyclic lactone creatinine (m/z 114).

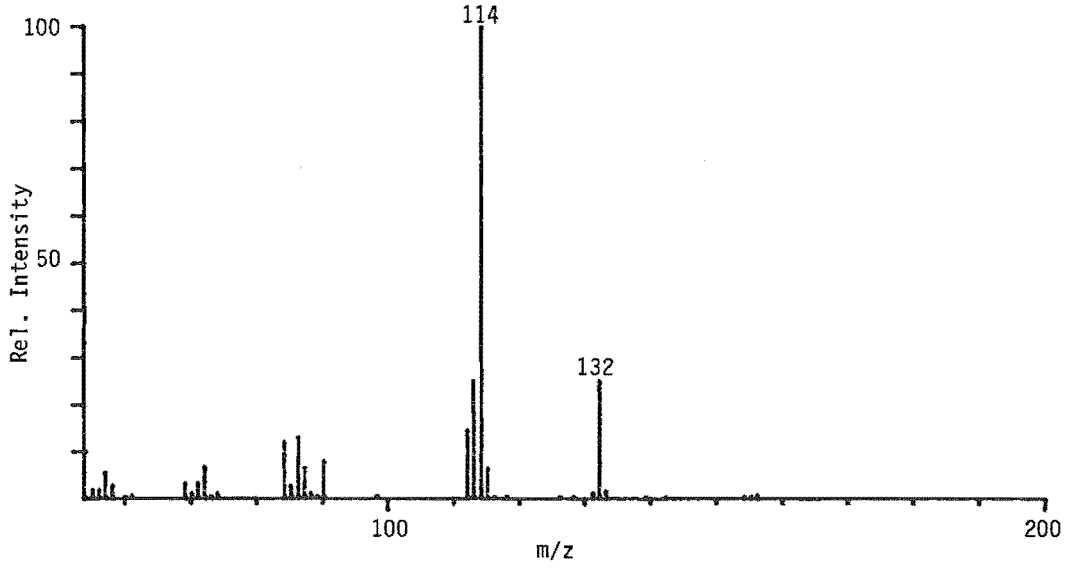


Figure 23. DCI mass spectrum of creatine

5.6 In-Beam Operating Parameters

The appearance of an in-beam mass spectrum is very dependent on experimental conditions. Spectra recorded using non-metallic probes, where the temperature of the source block determines the heating rate of the probe tip, have a protonated molecular ion intensity which is very sensitive to changes in both the source temperature and the position of the probe tip relative to the electron beam axis. Higher source temperatures have been reported to give a better relative abundance of this ion[132,139]. The optimal position for the probe tip has been found to be on[139], and 2.5 mm from[117,132] the electron beam axis. Spectra obtained using metallic (directly heated) probes have been recorded with the source temperature at normal[135] and at lower-than normal[138,151] temperatures. The probe tip has been positioned directly in the electron beam[124] in DEI experiments, and at various distances from it[120,135,151,143], including at the boundary of the ionisation chamber[138], in DCI experiments. In the latter case centering of the probe tip in the source was found to cause increased sample decomposition.

In this work DEI and DCI mass spectra were recorded at an ion source temperature of ca. 190^{o}C. This temperature (the normal source operating temperature) allowed the instrument to be used without delay to also record spectra for samples admitted to the ion source via the conventional solids probe inlet or the GC inlet.

The effect of the probe insertion distance during DCI and DEI operation was investigated as follows:

Spectra of creatine were recorded with the probe tip 0, 2.5, 5, 7.5, 10 and 15 mm from the electron beam axis while the mass spectrometer was operating under CI conditions with methane reagent gas. The relative intensity of the protonated molecular ion was determined at each distance by measuring the ratio $(M+H)^+/(M-OH)^+$. The results, presented in Figure 24, show that the abundance of the $(M+1)^+$ ion is strongly dependent upon the position of the probe tip in the ion source. The maximum value for the ratio of $(M+H)^+/(M-OH)^+$ was obtained when the probe tip was 2.5 mm from the electron beam axis. Spectra of creatine were also recorded with the probe tip 0, 2.5, 5, 7.5, 10 and 15 mm from the electron beam while the mass spectrometer was operating under EI conditions. An $(M+1)^+$ ion was only observed when the probe tip was retracted 2.5 mm.

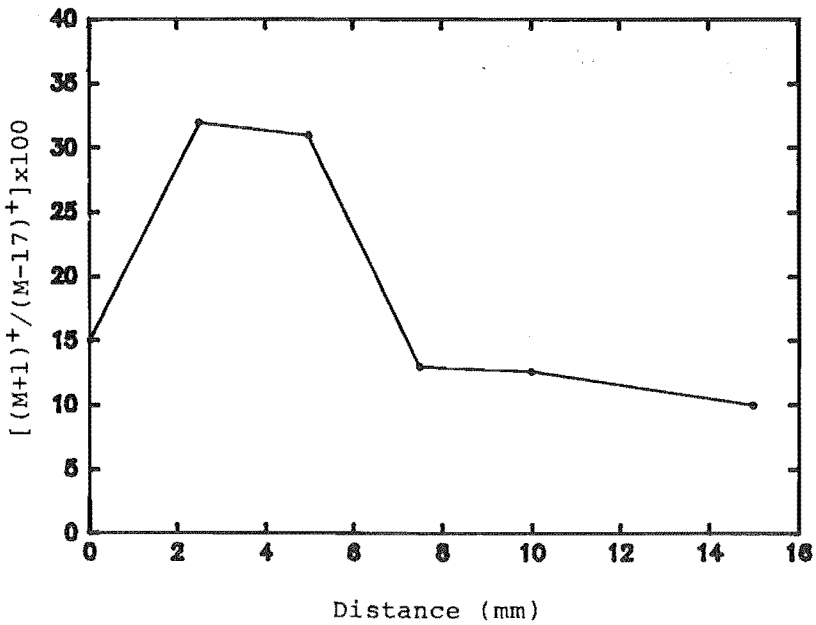


Figure 24. Variation of creatine $(M+1)^+/(M-17)^+$ ratio with distance from the ion beam axis to DCI probe tip.

5.7 Selected DEI and DCI Mass Spectra

In-beam mass spectra for seven compounds of biological interest which have not previously been studied by DEI or DCI-MS are now presented. These spectra show that the in-beam technique is a viable alternative to other ionisation methods for obtaining molecular weight (and other) information for "difficult" samples.

5.7.1 Barbitone and Amobarbitone

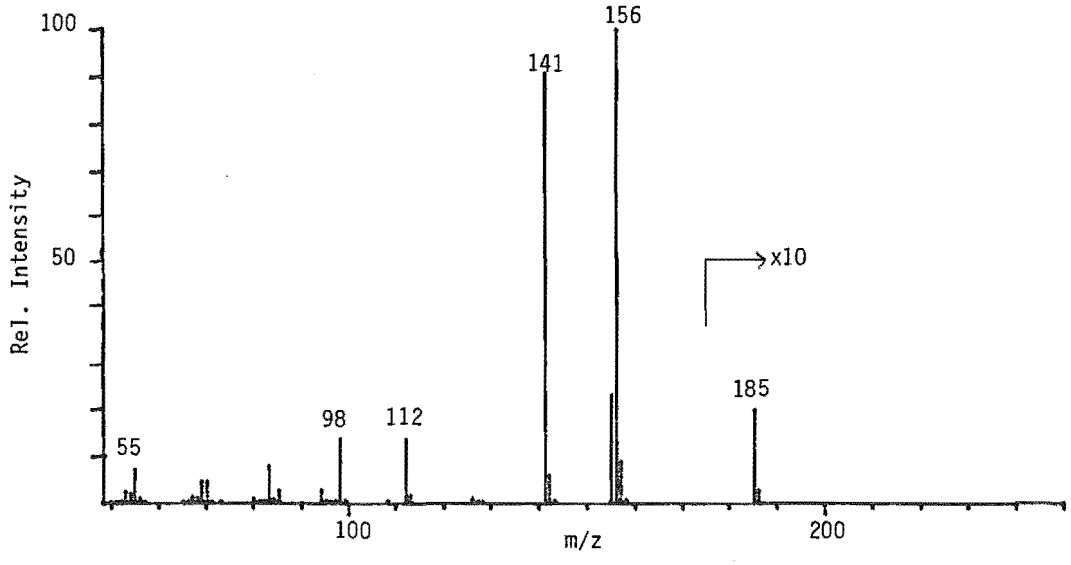
These two compounds are derivatives of barbituric acid. Both have sedative and hypnotic properties and, like many barbiturates, they show no molecular ion in their conventional EI mass spectra[152], (although intense $(M+1)^+$ ions are seen in their CI spectra[153]).

DEI spectra for barbitone and amobarbitone are presented in Figure 25. The spectra resemble very closely the corresponding EI spectra[152] but in addition show low intensity $(M+1)^+$ ions.

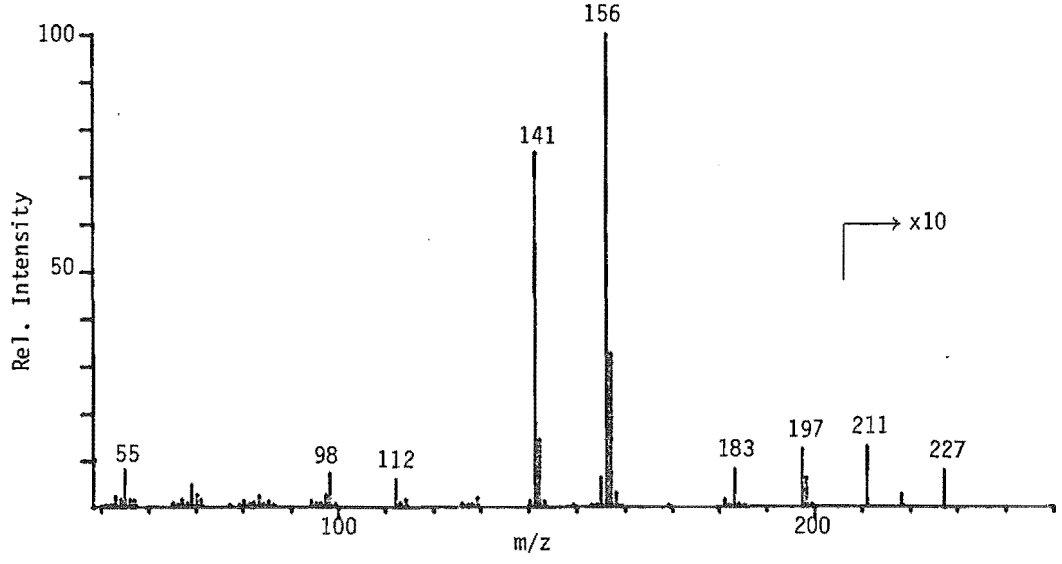
5.7.2 Meprobamate and Ephedrine

Meprobamate is the prototype of the medicinal carbamates and is frequently employed as a sedative, anti-anxiety agent and muscle relaxant. Ephedrine, as the ℓ-isomer, is a naturally occurring sympathomimetic amine that is used as a nasal decongestant and bronchodilator. The DEI spectra for meprobamate and ephedrine hydrochloride are presented in Figure 26. The EI spectra for both compounds[152] show no ions characteristic of molecular weight but the DEI spectra both show $(M+1)^+$ ion peaks.

Figure 25. DEI mass spectra of barbitone and amobarbitone.

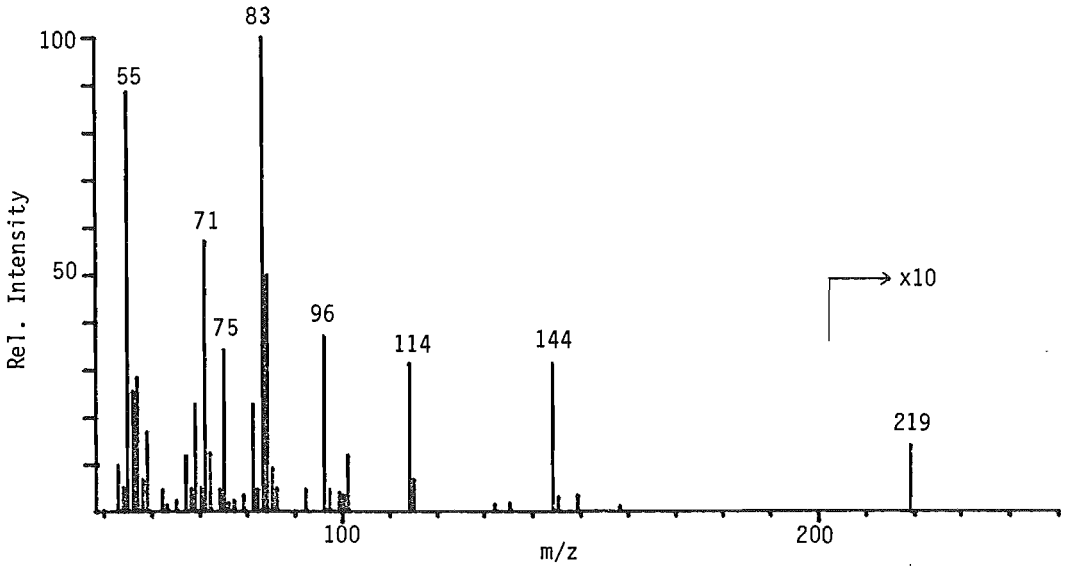


(a) DEI mass spectrum of barbitone

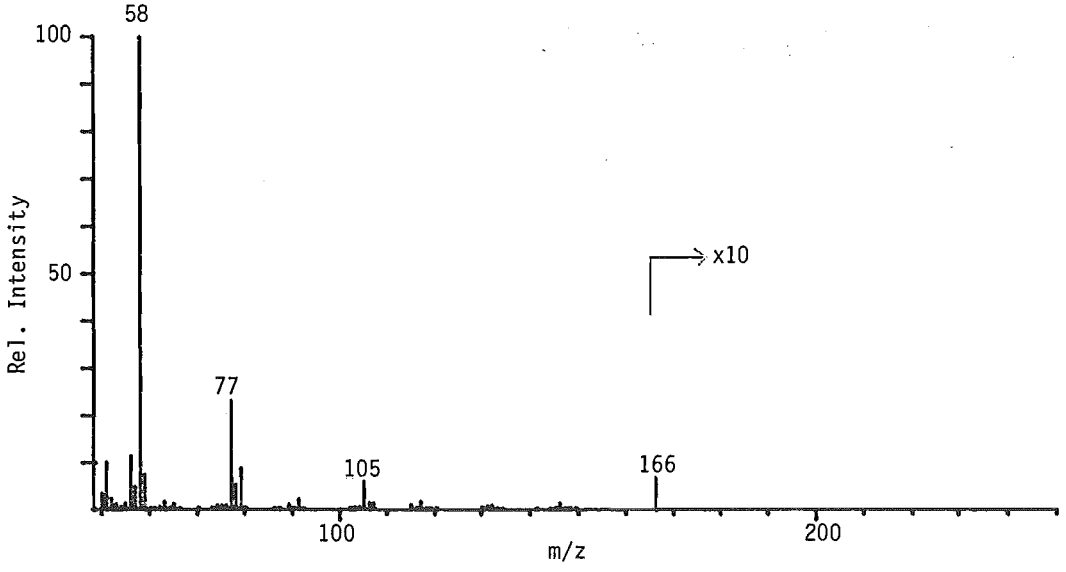


(b) DEI mass spectrum of amobarbitone

Figure 26. DEI mass spectra of meprobamate and ephedrine hydrochloride.



(a) DEI mass spectrum of meprobamate



(b) DEI mass spectrum of ephedrine hydrochloride

5.7.3 Discorhabdin C and Discorhabdin A

Two cytotoxic pigments named Discorhabdin C and Discorhabdin A (Figure 27) have recently been isolated from sponges of the genus Latrunculia du Bocage by the marine chemistry group at Canterbury University. These compounds, which are potential antitumour agents, were submitted for mass spectral analysis on the HP5982A.

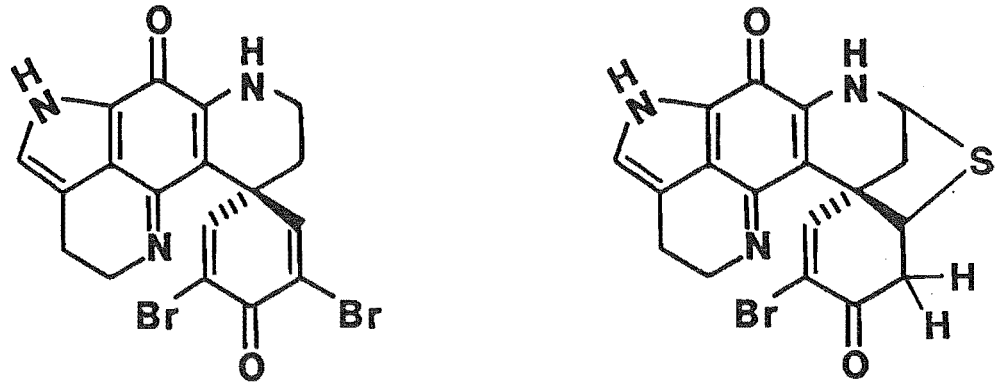


Figure 27. Structures for Discorhabdin C and Discorhabdin A.

The conventional EI spectrum of Discorhabdin C hydrochloride is presented in Figure 28. It shows an intense molecular ion cluster ($^{m}/z$ 461/463/465), and one significant fragment ion ($^{m}/z$ 147/149). All attempts to record conventional EI and CI spectra for Discorhabdin A hydrochloride were unsuccessful due to extensive decomposition of the sample as it was heated. The DCI/NH_3 spectrum of Discorhabdin A hydrochloride, recorded over a limited mass range, is presented in Figure 29. The spectrum shows an $(M+1)^+$ ion ($^{m}/z$ 416/418) and a fragment ion ($^{m}/z$ 384/386) corresponding to $(MH-S)^+$.

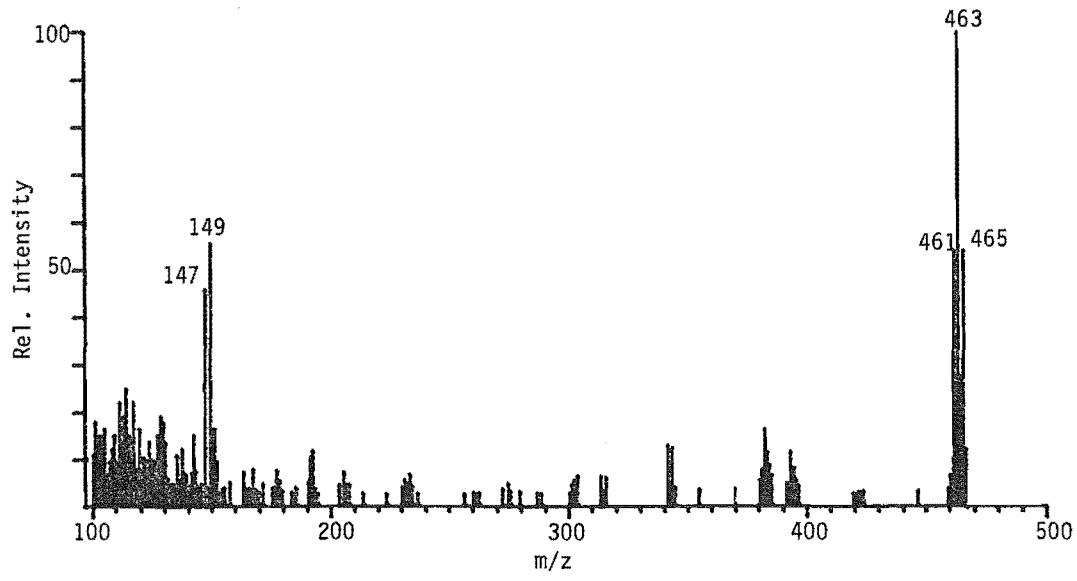


Figure 28. EI mass spectrum of Discorhabdin C hydrochloride

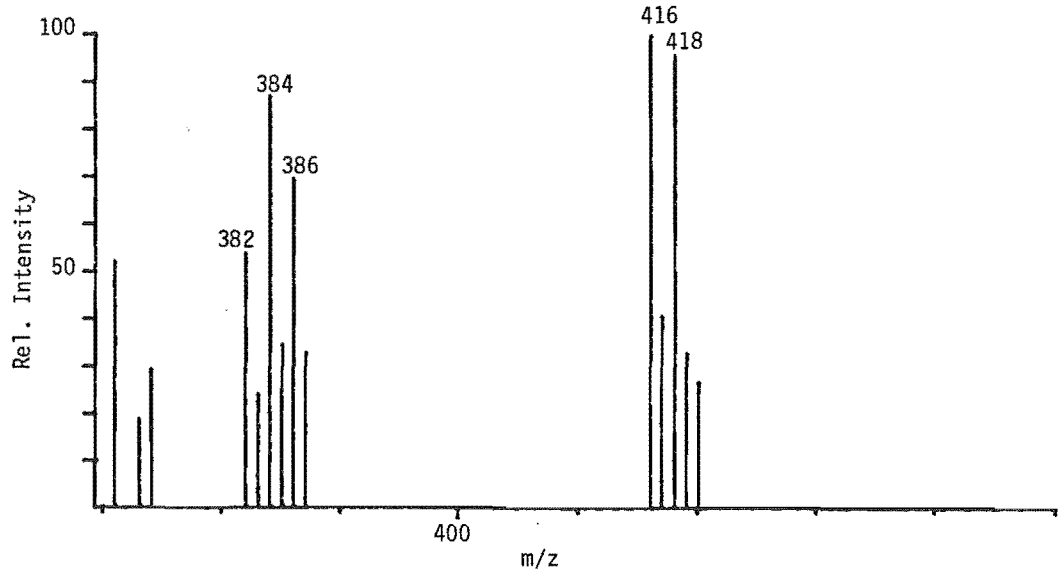


Figure 29. Partial DCI(NH_3) mass spectrum of Discorhabdin A hydrochloride

The utility of the in-beam technique in providing molecular weight information is further illustrated by FAB-MS results for Discorhabdin A hydrochloride. The FAB spectrum of this compound, recorded in a glycerol matrix, is presented in Figure 30. It shows peaks ($^{m}/z$ 418/420) resulting from in-situ reduction of the sample[154], making a priori interpretation of the spectrum difficult.

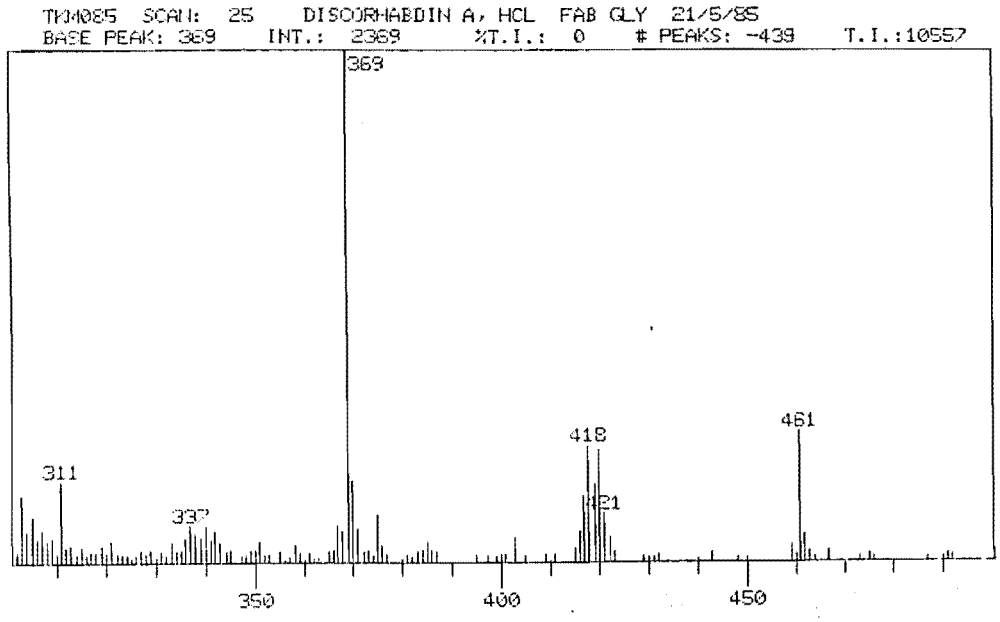


Figure 30. FAB (argon) mass spectrum of Discorhabdin A hydrochloride in glycerol. (Courtesy of Dr P.T. Holland, Ruakura Plant Research Station, Hamilton N.Z.)

5.7.4 Morphine-3-glucuronide

This compound has been discussed in Section 2.4.1 . The ammonia CI mass spectrum of the underivatised conjugate, presented in Figure 31, shows no ion characteristic of molecular weight. The two major peaks seen ($^{m}/z$ 286,303) correspond to the protonated aglycon $(Agly + 1)^+$ and $(Agly + NH_4)^+$ respectively. The DCI/NH_3 spectrum of morphine-3-glucuronide, presented in Figure 32, shows the $(M+1)^+$ ion ($^{m}/z$ 462).

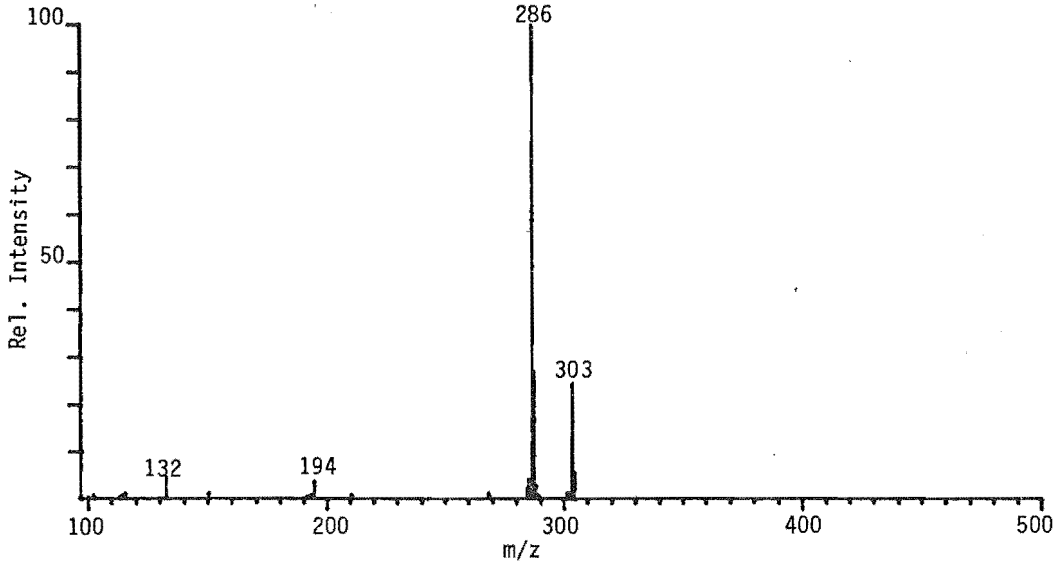


Figure 31. CI (NH_3) mass spectrum of morphine-3-glucuronide

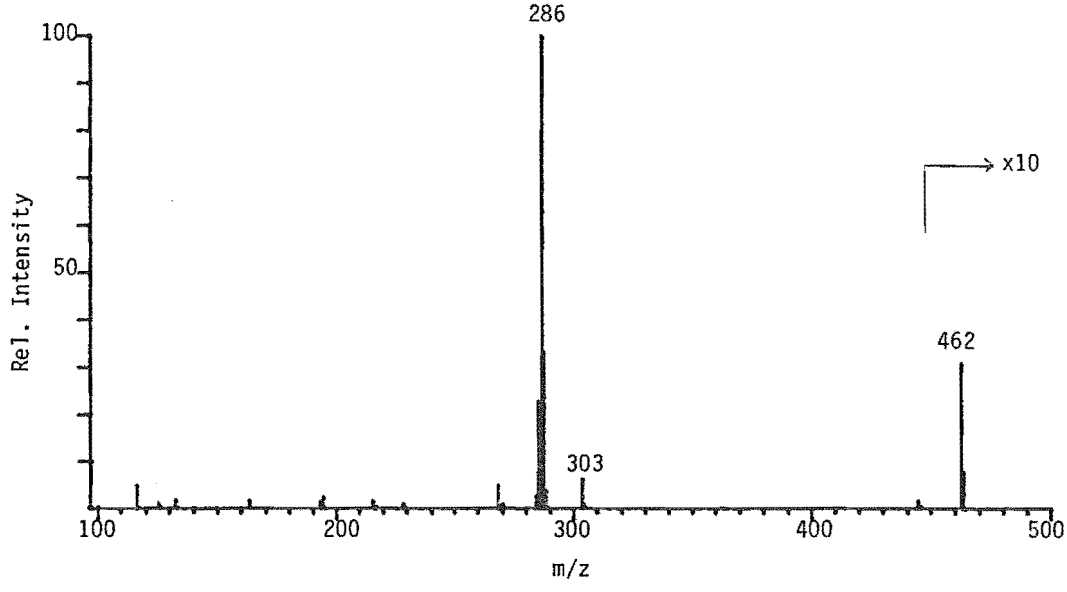


Figure 32. DCI (NH_3) mass spectrum of morphine-3-glucuronide

5.8 Conclusion

A Hewlett Packard 5982A GCMS has been successfully modified to allow it to be used to record in-beam mass spectra with a commercially available DCI probe.

CHAPTER 6

EXPERIMENTAL METHODS

6.1 General

Infrared spectra were recorded on a Shimadzu IR-27G spectrophotometer for liquid films and nujol mulls. ^{1}H n.m.r. spectra were obtained in deuterochloroform solvent with tetramethylsilane as an internal reference on a Varian T60 spectrometer. All chemical shifts are expressed as parts per million (ppm) downfield from TMS and are quoted as position (δ), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet) and relative integral.

Microanalyses were carried out by Professor A.D. Campbell and associates, University of Otago.

Melting points were determined in open capillaries and are uncorrected.

Weighings for standard solutions were performed on a Cahn model G electrobalance.

Yields reported for preparative procedures are those for the final purified product obtained after recrystallisation.

6.1.1 Reagents

Lithium aluminium deuteride (Merck) had an isotopic purity better than 98%. N,O-bis(trimethylsilyl)-acetamide and N,O-bis(trimethylsilyl)trifluoroacetamide (both Pierce Chemical Co., Rockford, Ill.) were analytical reagents. All solvents used for extractions were of analytical grade (AR) and were distilled twice in glass before being stored in

acid-washed glass jars with glass stoppers. All solvents used for preparative procedures were analytical grade or were purified (and dried when necessary) according to standard procedures[155,156]. "Ether" refers to diethylether. All drugs were supplied by the Chemistry Division of the Department of Scientific and Industrial Research (D.S.I.R.) or by the Department of Nephrology or the Department of Anaesthetics, Christchurch Hospital.

6.1.2 Biological Material

Post-mortem blood and post-mortem liver were supplied by the Chemistry Division of D.S.I.R. Out-dated plasma was supplied by the Blood Bank, Christchurch Hospital. All biofluid and tissue samples were stored frozen until needed.

6.1.3 Glassware

All glassware was initially cleaned by soaking in a chromic acid bath (70 mL saturated sodium dichromate solution and 2 L of concentrated sulphuric acid[157]) for several hours and subsequently rinsed thoroughly several times with hot water before being drained and oven dried. All glassware used for drug extraction procedures, including Reacti-vials, was silanised by soaking overnight in a solution of dimethyldichlorosilane in toluene (1:20) followed by a methanol wash. After use glassware used for extraction procedures was soaked in boiling "Decon 90" (Decon Laboratories Ltd., Hove, England) for about 5 h, thoroughly rinsed with distilled water and oven dried before being resilanised.

6.1.4 Instrumentation

The HP5982A GCMS used for this work has been described in Sections 1.2 and 1.3. The gas chromatograph was fitted with a 30 m x 0.32 mm i.d. fused silica BP-1 capillary column (SGE Pty. Vict. Australia). Helium was used as the carrier gas and the flow rate through the column was 3 mL min^{-1}. The temperature of the transfer oven was 250°C. The mass spectrometer ion source was operated in EI mode with an ionising energy of 50 eV. In CI mode the reagent gas pressure was optimised for the particular reagent gas used. The ion source temperature was ca. 190°C. Morphine analyses were performed using an OCI-3 on-column injector (SGE Pty.). Injections (1 µL) were made at an oven temperature of 120°C; after elution of the volatiles the oven temperature was increased rapidly to 280°C, giving a retention time for bis(trimethylsilyl)morphine of about 8 min. At the completion of each run the oven temperature was held at 300°C for 5 min. Benztropine and atropine analyses were both performed using a split/splitless injector (Chromalytic Technology Vict. 3150 Australia) operated in the split mode with a split ratio of 10:1. Injections (1 µL) were made at an oven temperature of 230°C, giving a retention time for benztropine of about 2 min. and for atropine of 3 min.

6.1.5 Mass Spectra

Mass spectra were recorded using a Datagraph 5-154 Recording Oscillograph (Bell and Howell, Pasadena, Ca.) connected to the GCMS. Ion intensities were determined by measuring peak heights with a ruler from the recorder paper. Mass spectra were normalised before being plotted.

6.2 Experimental Section Relating to Chapter 2

6.2.1 Preparative procedures

Morphine and N-trideuteriomethylnormorphine

The crude compounds[34] were purified by repeated recrystallisation from methanol:water followed by sublimation under vacuum.

Methyl(tetra-O-acetyl-β-D-glucopyranuronate)

Glucuronolactone (8.8 g) was treated with sodium methoxide and then acetic anhydride using the acid-catalysed acetylation procedure of Bollenback et al[36] to give a white solid which was recrystallised from ethanol. Yield: 5.08 g (27%); m.p. 176-177°(lit.[36] 176.5-178°).
M.s (CI/DIP ammonia), m/z: 394($M+NH_4^+$, 100%), 317 (53), 257(41).

Methyl(tri-O-acetyl-α-D-glucopyranosyl bromide)-uronate

Methyl(tetra-O-acetyl-β-D-glucopyranuronate)(2.5 g) was treated with 30% hydrobromic acid in acetic acid (10 mL) using the method of Bollenback et al[36] to give a white solid which was recrystallised from ethanol. Yield: 1.65 g (62%); m.p. 105-106° (lit.[36] 106-107°).
M.s (CI/DIP ammonia), m/z: 416/414 ($M+NH_4^+$, 71/100%), 317(28), 257(31).

Morphine-(3β-D-glucopyranosyl)-uronate

Morphine (0.10 g) was condensed with methyl-(tri-O-acetyl-α-D-glucopyranosyl bromide)-uronate (0.10 g) using the method of Berrang et al[35]. The crude product was

recrystallised twice from methanol. Yield: 40.2 mg (23%); m.p. 240-241°C(dec.) (lit.[35] 240-242°(dec); ^{1}H n.m.r. : Satisfactory spectra could not be obtained; I.R. (KBr): ν_{max} = 3610, 1633, 1503 and 14.56 cm^{-1}; M.s.(EI/DIP, as penta-trimethylsilyl derivative), m/z: 821 (partial spectrum only).

6.2.2 Preparation of Calibration Standards

Methanol stock solutions of D_0- and D_3-morphine were prepared in silanised volumetric flasks according to the scheme in Table 26. From these stock solutions standard mixtures of the following ratios were made up to 50 mL in silanised volumetric flasks using methanol solvent: 4:1 D_0:D_3 (800 µL and 200 µL), 2:1 (666 µL and 333 µL), 1:1 (500 µL and 500 µL, 1:2 (333 µL and 666 µL) and 1:4 (200 µL and 800 µL), using a 200-1000 µL adjustable micropipette.

The required standard mixtures (100 µL) were added to drug free plasma (1 mL), thoroughly mixed and left to stand for 1h before extraction and derivatisation using the method for unchanged morphine (Section 6.2.4).

Solution concn. (ng μL^{-1})	Mass of morphine (mg)	Final volume (mL)
10	0.5000	50
30	1.500	50
100	5.000	50
200	5.000	25

Table 26. Scheme for preparing morphine standard solutions

6.2.3 Determination of Solvent Extraction Efficiency

An appropriate amount of D_0-morphine was added to aliquots (1 mL) of drug-free plasma. The spiked samples were thoroughly mixed, left to stand for 1 h and then extracted according to the procedure for unchanged morphine (Section 6.2.4) but with the following change: D_3-morphine was added to the dry residue just prior to derivatisation.

6.2.4 Extraction of Morphine from Serum and Plasma

(a) Unchanged Morphine

An appropriate amount of D_3-morphine was added to serum (or plasma) (1 mL) in a 10 mL silanised-glass centrifuge tube having a ground glass stopper, thoroughly mixed and left to stand for 1 h. The serum (or plasma) was mixed with acetone (6 mL), centrifuged for 5 min, the organic layer transferred to a clean 10 mL centrifuge tube and the acetone evaporated at 70-80°C under nitrogen. The dry residue was taken up in 0.01 M hydrochloric acid (1 mL) and washed with benzene (2 x 4 mL). The aqueous phase was adjusted to pH 8-9 by addition of 1 M sodium hydroxide, buffered to pH 8.9 with borate buffer (1 mL) and extracted twice with isopropyl alcohol-dichloroethane (3:10 v/v) on a vortex mixer. The combined organic phase was reduced in volume, transferred to a 1 mL Reacti-vial (Pierce Chemical Co., Rockford, Ill.) and evaporated to dryness at 70-80°C under nitrogen. The residue was reacted with bis(trimethylsilyl) acetamide or bis(trimethylsilyl)trifluoroacetamide (50 µL) in a sealed vial for 1 h at 60°C, and the resulting mixture used directly for GCMS analysis.

(b) Total Morphine

An appropriate amount of D_3-morphine was added to serum (or plasma) (1 mL) in a 10 mL silanised glass centrifuge tube having a ground glass stopper, thoroughly mixed and left to stand for 1 h. The serum (or plasma) was buffered at pH 5.0 with ca. 0.2 M acetate buffer (1 mL), 2000 Fishman Units of β-glucuronidase (Sigma G-0750) added and the solution incubated in a water bath at 37°C for 24h. The procedure for unchanged morphine was then followed.

6.3 Experimental Section Relating to Chapter 3

6.3.1 Preparative procedures

Bromodiphenylmethane

Benzhydrol (16.80 g) was treated with phosphorus tribromide (2 mL) using the method of Claisan[76] to give a white solid which was purified by vacuum distillation (0.5 mm, 120°C). Yield: 16.0 g (80%); m.p. 42-43°C (lit.[76] 43-45°C).

Benztropine hydrobromide

Tropine (5.27 g) was reacted with bromodiphenylmethane (10 g) using the method of Weijlard[75]. Benztropine was isolated as its hydrobromide and recrystallised from ethanol. Yield: 4.80 g (33%); m.p. 258.5-260°C (lit.[75] 250-251°C).

Benztropine base

Benztropine hydrobromide (4.60 g) was dissolved in water (20 mL), the solution was basified with sodium

carbonate and then extracted with chloroform (4 x 50 mL). The combined chloroform extracts were dried over magnesium sulphate, filtered (Whatman phase separating paper) and the solvent removed under vacuum to give a pale yellow viscous oil. Yield: 3.41 g (94%); ^{1}H n.m.r. ($CDCl_3$) 1.75-2.15, m, 8H, ring CH_2; 2.22, s, 3H, CH_3; 2.90-3.40, m, 2H, CHNCH; 3.40-3.70, m, 1H, HCO; 5.38, s, 1H, OCH; 7.25, m, 10H, aromatic H; M.s. (CI/DIP ammonia), M/z: 308 ($M+H^+$, 100%), 167 (38), 140 (14), 124 (11), 110 (40).

N-(2,2,2-trichloroethoxycarbonyl)-norbenztropine

To a mixture of benztropine base (3.23 g) and potassium carbonate (0.05 g) in refluxing benzene (25 mL) was added 2,2,2-trichloroethylchloroformate (1.6 mL) dropwise over 0.5 h. Reflux was then continued for a further 2.5 h. The solution was allowed to cool, washed twice with sodium hydroxide (1M), once with water and dried over magnesium sulfate. After filtering, the solvent was removed under vacuum to leave a white solid which on trituration with pentane:dichloromethane gave white crystals. Yield: 1.44 g (29%); m.p. 121-122°C; (Found: C, 59.0; H, 5.2; N, 2.8; Cl, 22.7. $C_{23}H_{24}NO_3Cl_3$ requires C, 59.1; H, 5.1; N, 2.9; Cl, 22.5%); ^{1}H n.m.r. ($CDCl_3$) 1.70-2.40, m, 8H, ring CH_2; 3.55-3.90, m, 1H, HCO; 4.15-4.60, broad signal, 2H, CHNCH; 4.60-4.95, m, 2H, $COOCH_2$; 5.45, s, 1H, OCH; 7.28, m, 10H, aromatic H; M.s.(CI/DIP ammonia), m/z: 470/468, ($M+H^+$, 18/16%), 308(34), 293(26), 182(11), 167(100).

N-Trideuteriomethylnorbenztropine hydrobromide

A solution of N-(2,2,2-trichloroethoxycarbonyl)-norbenztropine (0.25 g) in dry tetrahydrofuran (10 mL) was added dropwise to a stirred slurry of lithium aluminium deuteride (0.225 g) and tetrahydrofuran (40 mL) and the reaction mixture was refluxed for 74 h. After cooling the reaction was quenched by the cautious addition of several drops of saturated sodium sulphate solution and filtered. Removal of the solvent under vacuum gave a pale yellow viscous syrup which was dissolved in ether (10 mL) and treated with 40% hydrobromic acid (0.1 mL). The mixture was shaken vigorously and allowed to stand for 1 h at room temperature. The resulting white precipitate was removed by filtration and recrystallised from water. Yield: 0.085 g (41%); m.p. 259-260°C.

N-Trideuteriomethylnorbenztropine base

N-Trideuteriomethylnorbenztropine hydrobromide (0.060 g) was dissolved in water (5 mL), the solution was basified with sodium carbonate and then extracted with chloroform (4 x 5 mL). The combined chloroform extracts were dried over magnesium sulphate, filtered (Whatman phase separating paper) and the solvent removed under vacuum to give a pale yellow viscous oil. Yield: 0.042 (88%), ^{1}H n.m.r. ($CDCl_3$) 1.75-2.28, m, 8H, ring CH_2; 2.90-3.40, m, 2H, CHNCH; 3.40-3.70, m, 1H, HCO; 5.37, s, 1H, OCH; 7.26, m, 10H, aromatic H; M.s.(CI/DIP ammonia), m/z: 311 ($M+H^+$, 100%), 167(34).

6.3.2 Preparation of Calibration Standards

Methanol stock solutions of D_0- and D_3-benztropine were prepared by weighing 2.500 mg of D_0- or D_3-benztropine into a silanised volumetric flask (50 mL) and making up to the mark with methanol. From these stock solutions standard mixtures of the following ratios were made up to 50 mL in silanised volumetric flasks using methanol solvent: 4:1 D_0:D_3 (800 µL and 200 µL), 2:1 (666 µL and 333 µL), 1:1 (500 µL and 500 µL, 1:2 (333 µL and 666 µL) and 1:4 (200 µL and 800 µL), using a 200-1000 µL adjustable micropipette.

The required standard mixtures (100 µL) were added to drug free blood (1 mL), thoroughly mixed and left to stand for 1h before extraction using the method for benztropine.

6.3.3 Determination of Solvent Extraction Efficiency

(a) Blood

An appropriate amount of D_0-benztropine was added to aliquots (1 mL) of drug-free blood. The spiked samples were thoroughly mixed, left to stand for 1 h and then extracted according to the procedure for benztropine in blood. (Section 6.3.4) but with the following change: D_3-benztropine was added just prior to evaporation of the organic solvent.

(b) Liver

An appropriate amount of D_0-benztropine was added to samples of homogenised liver. The spiked samples were then extracted according to the procedure for benztropine in

liver (Section 6.3.5) but with the following change: D_3-benztropine was added just prior to evaporation of the organic solvent.

6.3.4 Extraction of Benztropine from Post-mortem Blood

An appropriate amount of D_3-benztropine hyrobromide was added to post-mortem blood (1 mL) in a 10 mL silanised glass centrifuge tube having a ground glass stopper, thoroughly mixed and left to stand for 1 h. The blood was made alkaline (pH 11-12) with 0.5 M sodium hydroxide (360 µL) and extracted twice with 1-chlorobutane (2 x 5 mL) on a vortex mixer for 2 min. The combined organic phase was reduced in volume to 2 mL and extracted into 0.05 M suphuric acid (1 mL) in the same manner as before. The upper organic phase was discarded and the aqueous phase washed with 1 mL of extraction solvent before re-alkinisation with 0.5 M sodium hydroxide (220 µL). The extraction was then repeated as before with 5 mL of extraction solvent. The solvent was evaporated to dryness at 40-50°C under nitrogen and the residue reconstituted in ethanol (50 µL). The centrifuge tube was stoppered and the lower portion of the tube heated to 40-50°C in a hot block for several minutes to reflux the ethanol and wash down the inside of the tube. The sample was cooled and used directly for GCMS analysis.

6.3.5 Extraction of Benztropine from Post-mortem Liver

Liver (10 g) was blended with Tris base (40 mL) and crystalline subtilisin (0.2 g) in a Waring electric blender. The resulting suspension was transferred to a 100 mL conical flask, covered with a watch glass and heated with stirring

at 50-60°C for 1 h. An aliquot (1 mL) of the cooled liver digest was then extracted with 1-chlorobutane using the procedure for post-mortem blood.

6.4 Experimental Section Relating to Chapter 4

6.4.1 Preparative Procedures

N-Ethoxycarbonylnortropine

Tropine (2.50 g) was treated with ethylchloroformate (20 mL) using the method of Fung and DeGraw[98]. The crude product was recrystallised from ether to give a white solid. Yield: 1.80 g (51%); m.p. 72-75°C (lit.[96] 74-78; I.R. (nujol): ν_{max} 3420 (OH), 1680 cm^{-1} (C=O); ^1H n.m.r. ($CDCl_3$) 1.35-1.75, m, 3H, CH_3; 1.75-2.45, m, 8H, ring CH_2; 4.0-4.90, m, 5H, $COOCH_2$, CHNCH, HCO; M.s. (CI/DIP ammonia), $^m/_z$: 200 ($M + H^+$,100%).

N-Trideuteriomethylnortropine.

N-Ethoxycarbonylnortropine (1.76 g) was reduced with lithium aluminium deuteride using the method of Fung and DeGraw[98] to give a pale yellow viscous oil. Yield: 0.73 g (57%); I.R. (nujol): ν_{max} = 3320, (OH), 2200, 2070 cm^{-1} (C-D); ^1H n.m.r. ($CDCl_3$): 1.40-2.35, m, 8H, ring CH_2; 2.90-3.40, broad signal, 2H, CHNCH; 3.90-4.18, m, 1H, HCO; M.s.(CI/DIP ammonia), $^m/_z$: 145 ($M+H^+$, 100%).

Tropic acid.

Phenylacetic acid (19.75 g) was reacted with magnesium and isopropylchloride to form the corresponding Grignard derivative, which was then treated with formaldehyde and

hydrolysed to tropic acid, all by the method of Blicke[99]. The crude product was recrystallised from benzene. Yield: 12.58g (52%); m.p. 114-115^0 (lit.[99] 116-117); ^{1}H n.m.r. (D_6DMSO/$CDCl_3$): 3.40-4.20, m, 3H, CH, CH_2; 7.30, s, 5H, aromatic.

Acetyltropyl chloride

Tropic acid (3.0 g) was treated with acetyl chloride and then thionyl chloride using the method of Toomey and Riegel[100]. The acetyltropyl chloride was used immediately. Yield: 2.90 g (66%); ^{1}H n.m.r. (D_6DMSO/$CDCl_3$; 1.95, s, CH_3; 3.60-4.95, 3H, CH,CH_2; 7.32, s, 5H, aromatic.

N-Trideuteriomethylnoratropine.

N-Trideuteriomethylnortropine (0.70 g) was reacted with acetyltropyl chloride (1.40 g) using the method of Fung and DeGraw[98] to give a white solid which was recrystallised from acetone. Yield: 0.75 g (53%); m.p. 106-108^{o}C (lit.[98] 105-108); I.R. (Nujol): ν_{max} = 3500, 3320 (OH), 2200, 2070, 2050 cm^{-1}; ^{1}H n.m.r. ($CDCl_3$): 1.50-2.30, m, 8H, ring CH_2; 2.60-3.20, broad signal, 3H, OH, CHNCH; 4.90-5.10, m, 1H, HCO; 7.30, s, 5H, aromatic H.

6.4.2 Preparation of Calibration Standards

Stock solutions of D_0- and D_3-atropine were prepared by weighing 0.500 mg of D_0- or D_3-atropine into a silanised volumetric flask (10 mL) and making up to the mark with methanol. From these stock solutions std mixtures of the following ratios were made up to 5 mL in silanised

volumetric flasks using methanol solvent: 4:1 D_0:D_3 (800 µL and 200 µL), 2:1 (666 µL and 333 µL), 1:1 (500 µL and 500 µL, 1:2 (333 µL and 666 µL) and 1:4 (200 µL and 800 µL), using a 200-1000 µL adjustable micropipette. The std mixtures were used directly as calibration standards.

6.4.3 Preparation of Aqueous Plant Extract

The preparation of the aqueous plant extract is described in Section 4.6.

6.4.4 Extraction of Aqueous Plant Extract

An appropriate amount of D_3-atropine was added to the aqueous plant extract (1 mL) in a 10 mL silanised glass centrifuge tube having a ground glass stopper and the tube shaken. The extract was made basic (9-10) with aqueous ammonia solution and extracted twice with chloroform. The combined organic phase was evaporated to dryness at 70-80°C under nitrogen and the residue reconstituted in ethanol (100 µL). The centrifuge tube was stoppered and the lower portion of the tube heated to 40-50°C in a hot block for several minutes to reflux the ethanol and wash down the inside of the tube. The sample was cooled and used directly for GCMS analysis.

6.4.5 Soxhlet Extraction of Plant Material

Dried plant material (2.50 g) was extracted in a soxhlet apparatus with a mixture of 0.880 ammonia (60 mL), ethanol (120 mL) and ether (960 mL) for 24 h. The extract was allowed to cool, and then was filtered into a 2 L

volumetric flask and made up to the mark with additional ether. An aliquot (1 mL) of the extract was placed in a 10 mL silanised glass tube having a ground glass stopper, an appropriate amount of D_3-atropine added, and the tube shaken. The organic phase was extracted into 0.05 M sulphuric acid (1 mL) and the organic phase discarded. The aqueous phase was made basic (pH 9-10) with aqueous ammonia solution and extracted twice with chloroform. The combined organic phase was evaporated to dryness at 70-80^{o} under nitrogen and the residue reconstituted in ethanol (100 µL). The centrifuge was stoppered and the lower portion of the tube heated to 40-50^{o}C in a hot block for several minutes to reflux the ethanol and wash down the inside of the tube. The sample was cooled and used directly for GCMS analysis.

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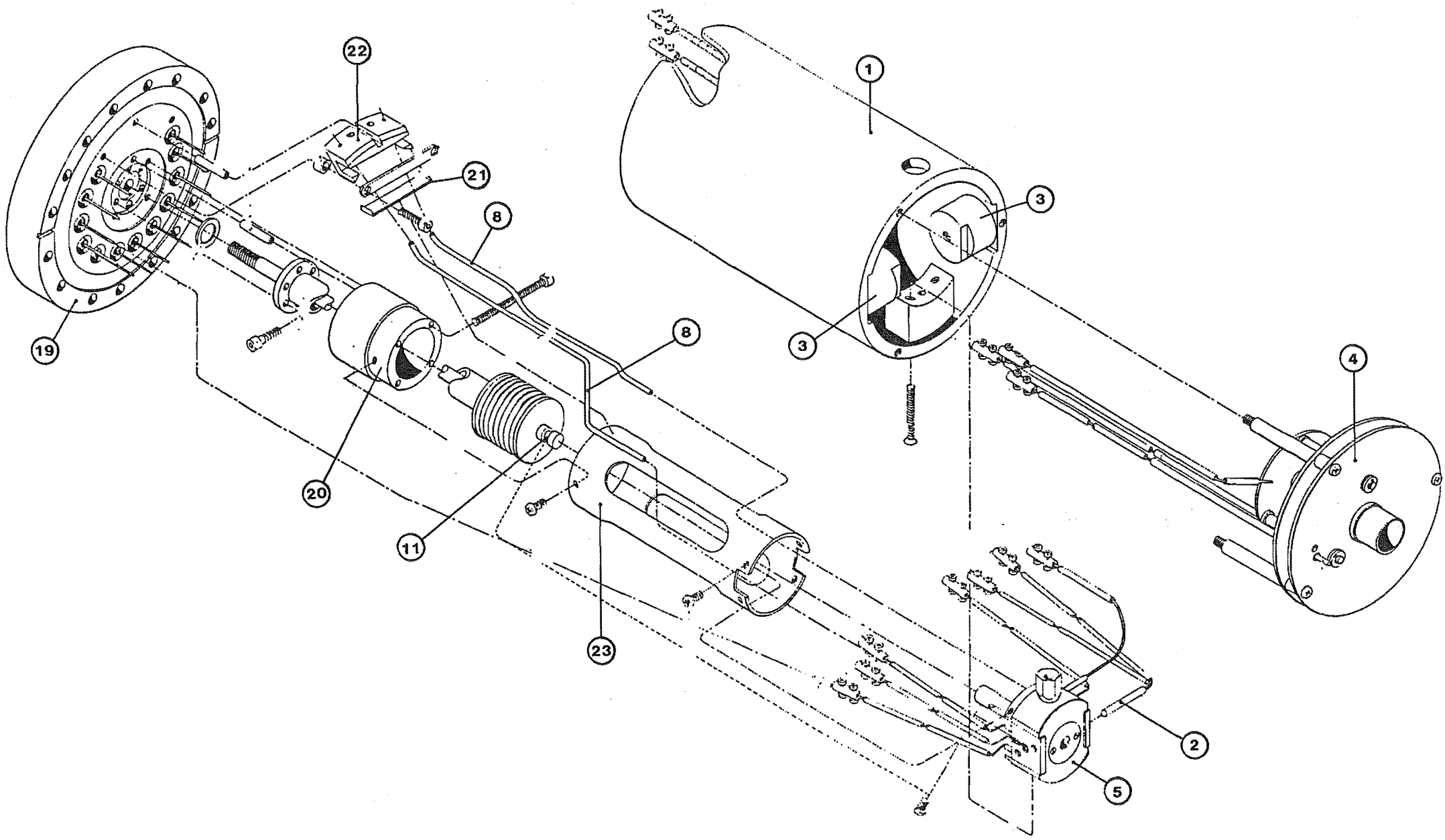


Figure 33. Exploded view of ion source

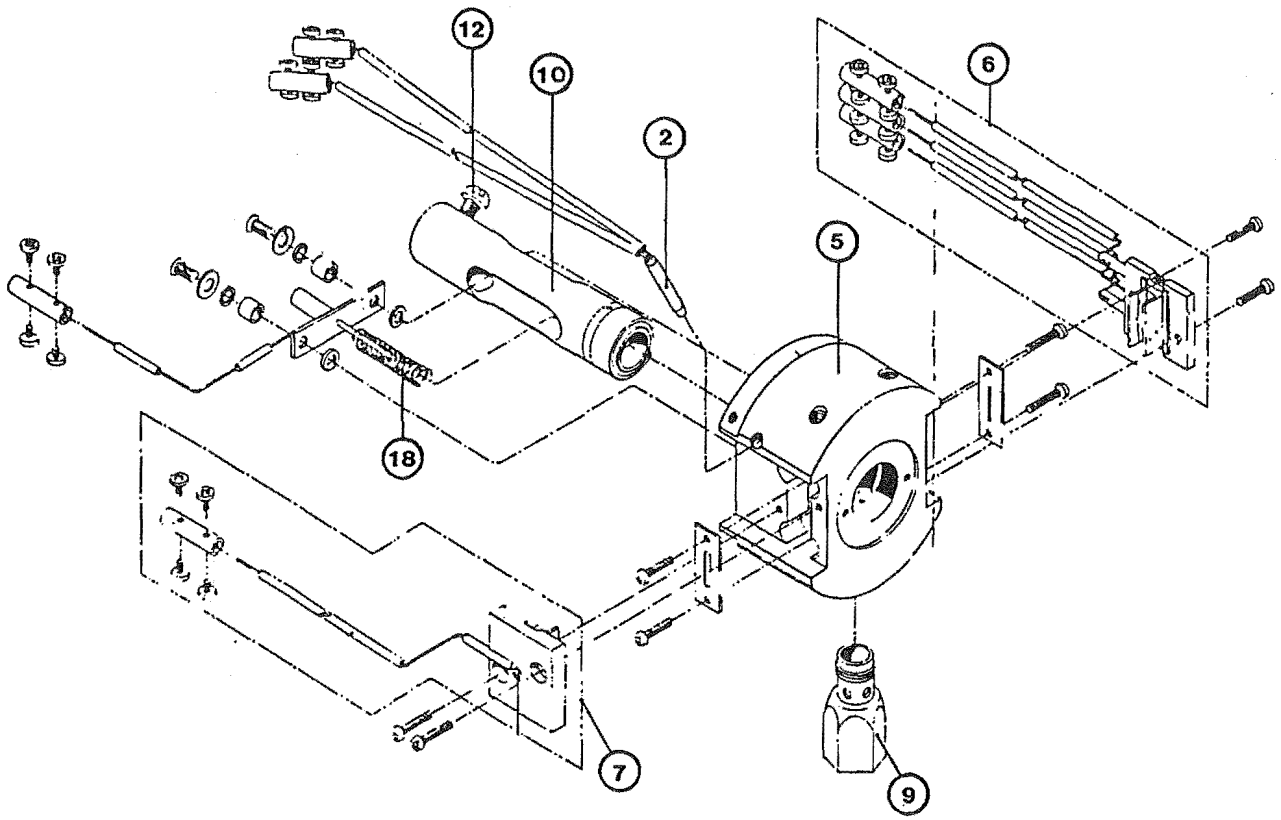


Figure 34. Exploded view of the ion source

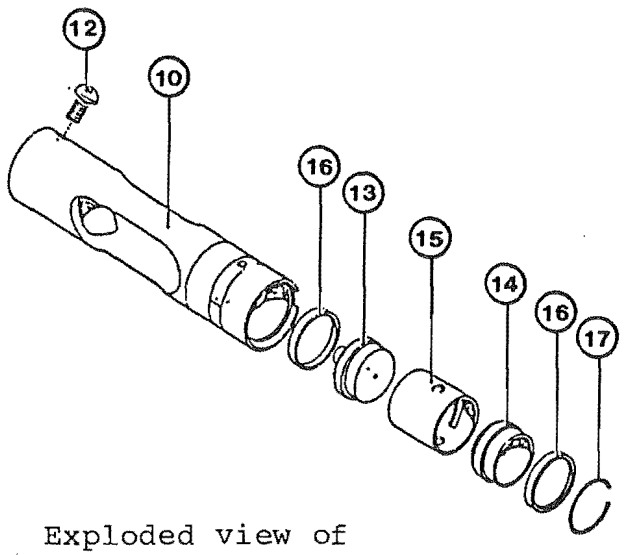
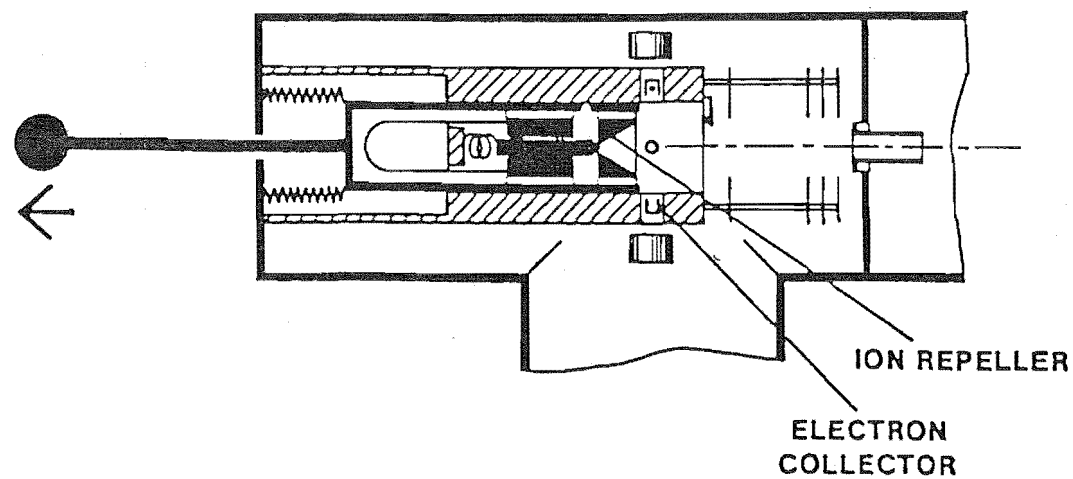


Figure 35. Exploded view of the plunger assembly

ELECTRON IMPACT CONFIGURATION



CHEMICAL IONIZATION CONFIGURATION

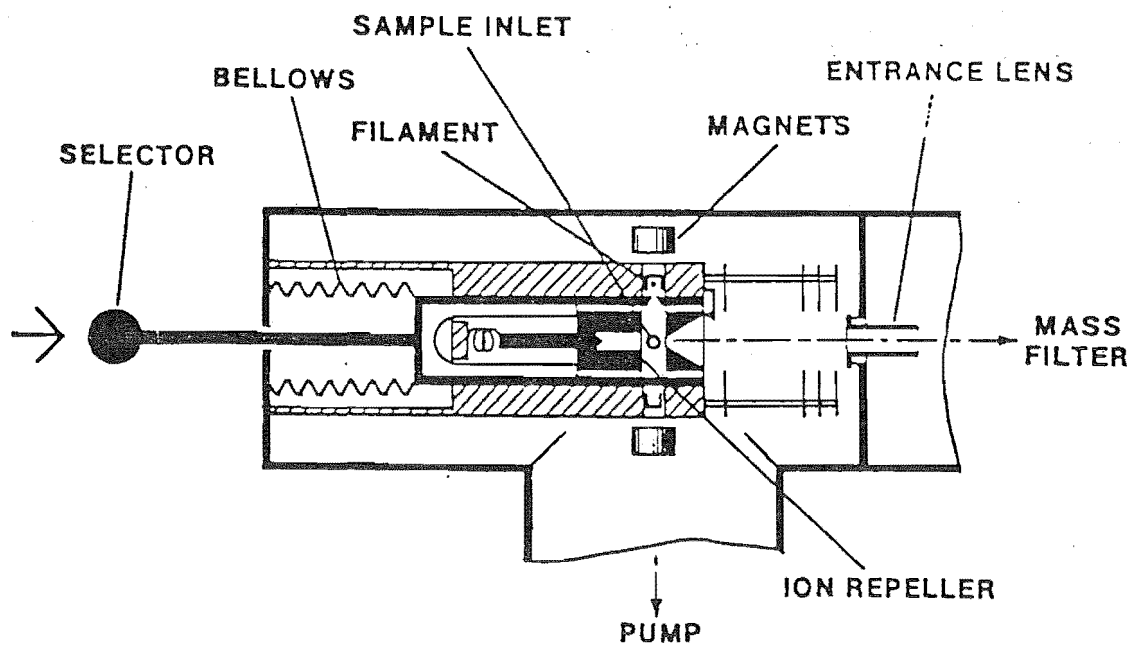


Figure 36. Ion source configurations

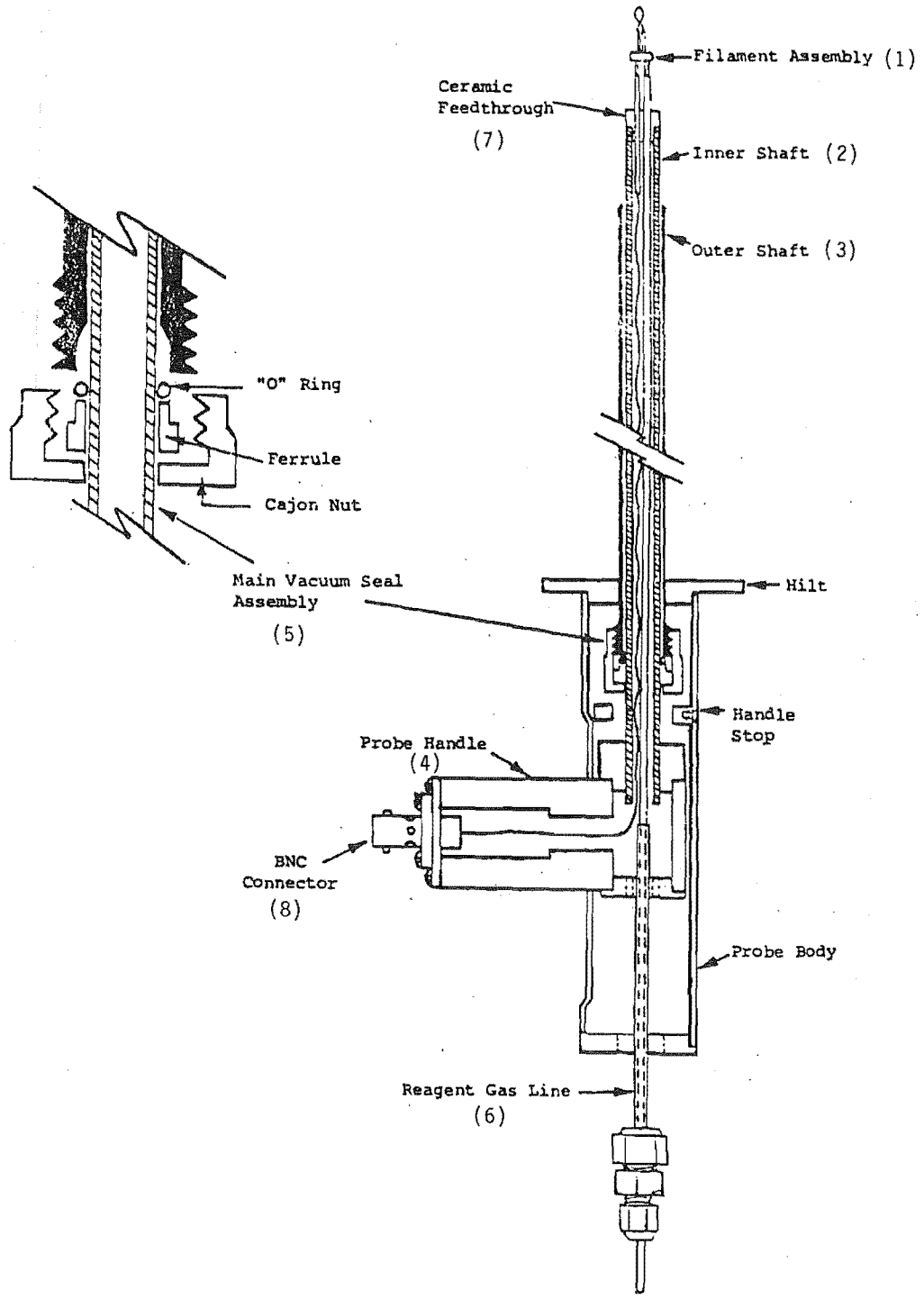


Figure 37. Cross section scale drawing of the DCI probe

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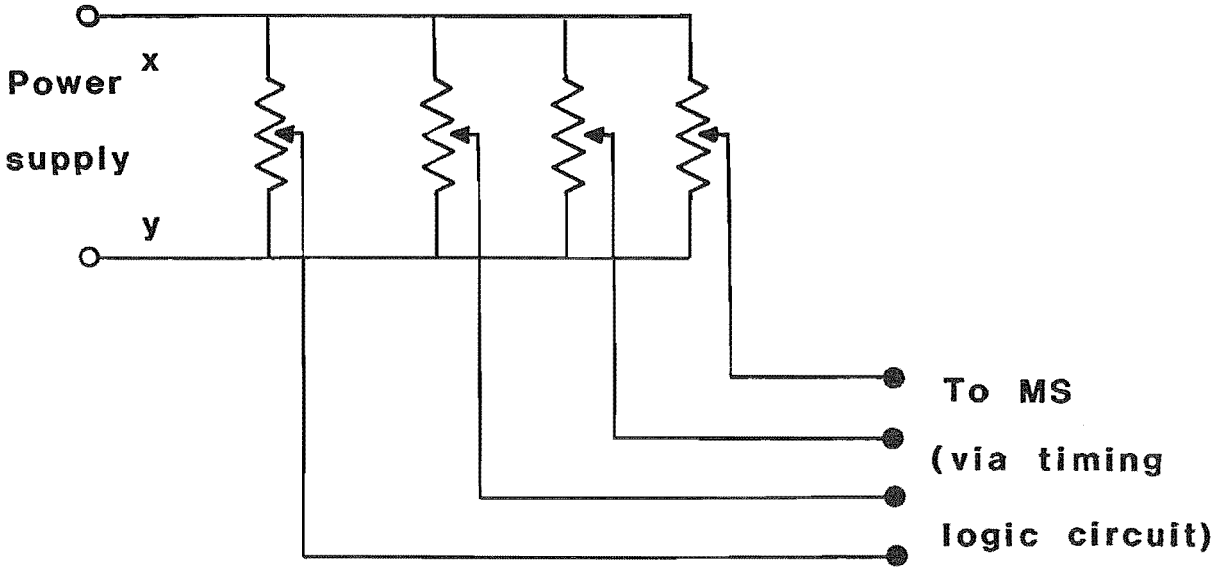
Appendix A

The tuning voltage supplied by each channel of the MID to control the mass spectrometer is adjusted using the following circuit.



The mass range covered by each potentiometer was reduced by inserting a 2 kilohmn resistor at "x" and a 500 ohm resistor at "y".

Appendix B

Note 1:

Drugs absorbed from the gastrointestinal tract into the bloodstream are carried first in the portal circulation to the liver where they may be metabolised. Loss of drug from the bloodstream on passage through the liver is termed the first-pass effect. In some cases, the first-pass effect may result in virtually complete elimination of the original drug before it gains access to the general circulation.

Note 2:

Haemodialysis is a procedure for removing waste products of metabolism from the blood when renal function is inadequate or has failed completely. Blood from the patient is passed through an artificial membrane surrounded by a dialysis fluid (the dialysate), and returned to the patient. Waste products such as urea, creatinine and uric acid diffuse out of the blood, across the dialyser membrane and into the dialysate. An anticoagulant is required to prevent the blood from clotting.

Note 3:

Haemodialysis requires repeated, reliable vascular access. This used to be by means of an external artificial arteriovenous shunt (Figure 38). During dialysis, the shunt is disconnected and the ends joined to the appropriate parts of the dialyser. Such shunts tended to clot and gave the patient no freedom to immerse the limb in water. The commonest access

in use today is a subcutaneous arteriovenous fistula (Figure 39). The radial artery in the non-dominant arm is joined, usually side to side, with the vein at the wrist so that the vein back up the arm increases in size and develops a thicker wall. This enlarged artery-like vein can be needled to provide access to the bloodstream, first to withdraw blood and then to return it after it has passed through the dialyser.

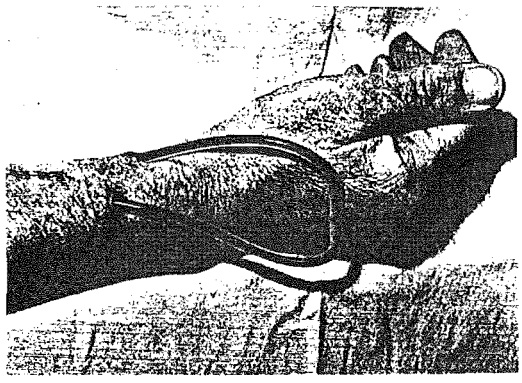


Figure 38. External arteriovenous shunt.

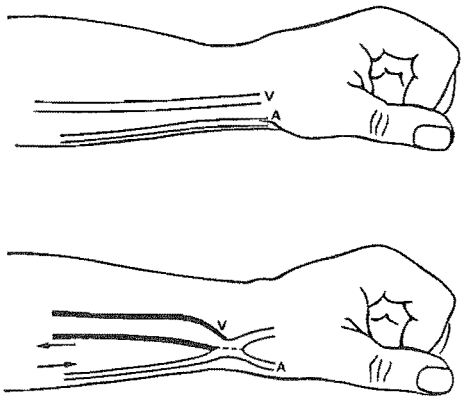


Figure 39. Subcutaneous arteriovenous fistula.

Note 4:

A drug that is secreted into the bile is presented again to the intestine and may be reabsorbed and passed again to the liver. This cycle of events is termed enterohepatic recirculation. The effect of this cycle is to increase the persistance of the drug in the body and, providing the concentrations of the drug at its sites of action are sufficiently high, to prolong its duration of action.

Appendix C

The equation of the weighted regression line which minimises the sum of the squares of the residual differences between the observed y values and the y values predicted from the regression line is given by $y = a + bx$, where b is the best estimate of the slope, given by

$$b = \frac{\Sigma UV}{\Sigma U^2}$$

and the best estimate of the intercept is given by

$$a = \bar{y} - b\bar{x}$$

where

$$w = \frac{1}{s^2}$$

$$\bar{x} = \frac{\Sigma wx}{\Sigma w} \quad \text{and} \quad \bar{y} = \frac{\Sigma wy}{\Sigma w}$$

$$\Sigma U^2 = \Sigma w(x-\bar{x})^2 = \Sigma wx^2 - \bar{x}^2\Sigma w$$

$$\Sigma V^2 = \Sigma w(y-\bar{y})^2 = \Sigma wy^2 - \bar{y}^2\Sigma w$$

and $$\Sigma UV = \Sigma w(x-\bar{x})(y-\bar{y}) = \Sigma wxy - \bar{x}\,\bar{y}\,\Sigma w.$$